INVESTIGATING COTRANSLATIONAL PROTEIN INTEGRATION INTO THE ENDOPLASMIC RETICULUM MEMBRANE

A Dissertation

by

PETER JOSEPH MCCORMICK

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2003

Major Subject: Biochemistry

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Approved as to style and content by:

Arthur E. Johnson (Chair of Committee)	Gregory D. Reinhart (Member)
John M. Scholtz (Member)	Gregory D. Reinhart (Head of Department)
Michael Kladde (Member)	

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ABSTRACT

Investigating Cotranslational Protein Integration into the Endoplasmic Reticulum Membrane. (December 2003)

Peter Joseph McCormick, A.B., Washington University in St. Louis

Chair of Advisory Committee: Dr. Arthur E. Johnson

During co-translational integration, the transmembrane (TM) sequence of a nascent membrane protein moves laterally into the ER lipid bilayer upon reaching the translocon. Our lab has previously shown that this movement is a multistep process, but it was not clear whether the observed photocrosslinking of the TM segment to translocon proteins resulted from specific interactions or simply from TM-translocon proximity. If the latter, the TM α-helix will be oriented randomly with respect to translocon proteins, whereas, if the former, a specific TM helix surface would face TRAM and/or Sec61α. Integration intermediates were prepared by in vitro translation of truncated mRNAs in the presence of a Lys-tRNA analog with a photoreactive moiety attached to the lysine side-chain. When photoadduct formation was monitored as a function of probe location within the TM α-helix, we found that the extent of photocrosslinking to TRAM and Sec61α was non-random. Thus, the TM sequence occupies a distinct location within the translocon, a result that

can only be achieved through protein-protein interactions that mediate the lateral movement, positioning, and integration of the TM sequence.

In the case of multi-spanning membrane proteins, it was unknown how multiple hydrophobic regions integrated into the ER membrane. By placing photoprobes within each of several TM domains of a multi-spanning membrane protein, we were able to determine at what stage of integration each TM segment was no longer adjacent to translocon proteins. Using this approach we were able to establish a mechanism of integration for multi-spanning membrane proteins co-translationally inserted into the ER membrane.

ACKNOWLEDGEMENTS

Great accomplishments are not achieved alone. I have had many people give me both advice and help over the years. First I would like to thank my parents, Shannon C. McCormick, Dr. Joseph B. McCormick and Dr. Susan P. Fisher-Hoch. My mother has taught me to be forgiving, caring, and helpful to those around me, traits I have used many times in both teaching the numerous students I have trained and in working with my colleagues. Pop and Sue have been my inspiration for both my professional career and my personal relationship. Their endless energy and dedication to both their craft and to each other is unique and a model.

Second, I would like to thank my wife, Dr. Joye E. Purser. She has taught me how to succeed in science and still keep a smile on my face. She has kept my life in balance and encouraged me over all obstacles; without her I surely would have stumbled. Third, I would like to thank my siblings, Anne McCormick, and Major Kit McCormick for their support and love over the years.

Graduate school is a character test and it is a test I could not have passed without the many classmates I had. Specifically, I would like to thank Dr. Matthew Champion and Dr. Pablo Sobrado for the late nights debating experiments and papers over a beer, a chess game, or a cup of coffee. The constant jousting fed my competitive spirit and helped to propel me along. Their friendship has never waned despite numerous

trials. They have taught me to never let anyone keep me down and to hold my head high.

I would next like to thank everyone in the Johnson lab who have taught me and provided for me throughout my tenure. Specifically, I would like to thank Yiwei Miao and Yuanlong Shao who have faithfully provided me with the materials I needed to perform my experiments year in and year out as well as Lisa Eubanks for always keeping me on track with deadlines and paperwork. Next, I would like to thank my committee members for the wonderful classes I had with them and for their guidance during my graduate career.

Finally, I would like to pay tribute to my boss and my friend, Dr. Arthur E. Johnson. His mix of patience and tough love has shown me what it takes to compete and succeed in this business. His knowledge knows no bounds. His ability to grasp the essentials of a problem and to explain it never ceases to amaze. His relentless desire for perfection, although often frustrating for those of us in the lab, always seemed to pay off and be the correct thing to do. Art, thank you, it has been an honor and a pleasure to work for you.

TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

FIGURE Page

CHAPTER I

INTRODUCTION

Protein Trafficking at the ER Membrane

In mammalian cells, the vast majority of ribosomes are found within the cytosol synthesizing polypeptide. Among this pool of ribosomes are two major populations, those ribosomes synthesizing cytosolic proteins and those translating proteins destined to be secreted from the cell. These latter ribosomes must be recognized and targeted to the Endoplasmic Reticulum (ER), the point of entry into the secretory pathway. Ribosomes destined for the ER are delineated by the polypeptides they are synthesizing. These polypeptides contain a signal within the first 15-30 amino acids of the nascent chain (Krieg et al.,1986; Kurzchalia et al., 1986), called a signal sequence (SS), that is recognized by a cytosolic ribonucleoprotein, called the signal recognition particle (SRP). SRP then targets the ribosome●nascent chain complexes to the ER membrane at sites termed translocons. Upon completion of synthesis, the ribosomes release from the ER membrane and return to the cytosol for another round of synthesis. This cycle of synthesis, targeting, and release of ribosomes is repeatedly constantly as the cell produces either soluble or integral membrane proteins for secretion (depicted in Fig. 1).

Those secretory proteins that are soluble are translocated completely

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This dissertation follows the style and format of Cell.

across the ER membrane. The mechanism for translocation has been studied extensively, (Johnson and van Waes, 1999; Rapoport et al., 1996; Schnell and Hebert, 2003; Walter and Johnson, 1994). Briefly, as mentioned above, cytosolic ribosomes translating secretory proteins are targeted to the ER membrane via binding of SRP to the SS of the nascent chain. Upon binding of SRP to the ribosome•nascent chain complex (RNC), elongation is arrested as the complex diffuses in the cytosol prior to encountering and binding to the ER membrane. At the membrane, SRP interacts with an integral membrane GTPase, the SRP receptor (SR), and this leads to the ribosome binding to the translocon, the release of SRP and SR from the ribosome, and the resumption of protein synthesis. It is at the translocon that the processes of translocation and integration diverge. Secretory proteins are translocated completely across the membrane into the lumen of the ER, while membrane proteins are integrated laterally into the lipid bilayer.

Single-Spanning Membrane Proteins

Most membrane proteins are inserted co-translationally into the membrane of the endoplasmic reticulum (ER) at translocons (reviewed in (Johnson and van Waes, 1999). Ribosomes are therefore synthesizing the nascent membrane proteins as they are being processed at the ER membrane, and these ribosomes are bound to translocons. The primary structural feature of

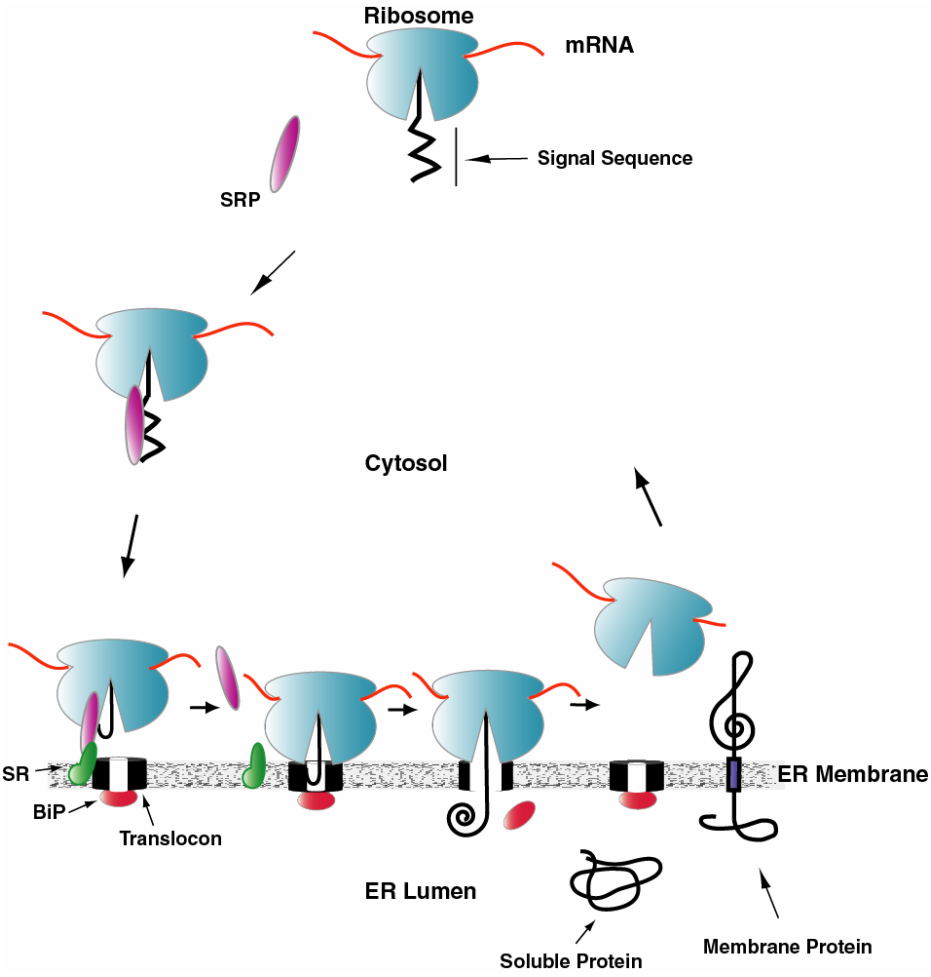

Figure 1 The Cycle of Ribosomes Synthesizing Secretory Proteins.

Cytosolic ribosomes synthesizing a nascent chain containing a signal sequence are recognized and bound by SRP. The ribosome-nascent chain complex is then targeted to the translocon where an interaction with SRP and SRP receptor (SR) leads to ribosome binding to the translocon and the insertion of the nascent chain into the translocon pore. When the nascent chain reaches ~70 amino acids in length, BiP is released from the lumenal end of the pore and translocation and are integration proceeds. After termination of translation, the pore is resealed on its lumenal side by BiP, and the ribosome is released into the cytoplasm for another round of synthesis (Adapted from Johnson and Van Waes, 1999).

the translocon is the aqueous pore that completely spans the membrane and through which nascent secretory proteins are translocated into the ER lumen (Crowley et al., 1994). Yet the presence of these holes does not compromise the permeability barrier of the ER membrane because ion movement through the pores is prevented either by ribosomes binding to the cytoplasmic side of the translocon pore (Crowley et al., 1994; Liao et al., 1997) or through the binding of BiP to the lumenal side of the membrane, either during the integration process (Haigh and Johnson, 2002) or after translation terminates and the ribosome leaves the translocon (Hamman et al., 1998).

The complexity and dynamic nature of translocon structure and function are particularly evident during nascent chain integration (Johnson and van Waes, 1999). The conversion of the operational mode of the translocon from the translocation of a secretory protein to the integration of a membrane protein requires communication between the translocon and the ribosome because the ribosome, not the translocon, first detects a transmembrane (TM) sequence in a nascent membrane protein (Liao et al., 1997). This information is then communicated through a very long signal transduction pathway from well inside the ribosome to the lumenal side of the ER membrane. Furthermore, a highly coordinated sequence of structural changes at the translocon are required to maintain the permeability barrier of the membrane while hydrophilic domains of a nascent membrane protein are alternately directed to the lumenal or the

cytoplasmic sides of the bilayer during integration (Haigh and Johnson, 2002; Liao et al., 1997).

In addition to controlling ion movement through the pore, the translocon must also regulate molecular movement in the plane of the membrane because the TM sequences in a nascent membrane protein must be identified and moved laterally from the aqueous pore into the hydrophobic interior of the ER membrane. Such a movement would require the protein components of the translocon to separate and thereby allow passage of a TM sequence through the translocon. Currently, it is widely believed that a nonpolar TM sequence in the aqueous translocon pore is recognized by its hydrophobicity and moved laterally into the interior of the ER bilayer where it is surrounded by phospholipids. The experimental support for this view of integration is the high efficiency of photocrosslinking of TM sequences to phospholipids when the TM sequences reach the translocon (Heinrich et al., 2000; Martoglio et al., 1995; Mothes et al., 1997). Photocrosslinking of nascent chains to translocon proteins was also observed, but the extent of such crosslinking was much less than to phospholipids. Based on the preponderance of photoadducts containing phospholipid, the crosslinks to translocon proteins were assumed to be random and non-functional. It was therefore concluded that the hydrophobic TM segment is exposed to the nonpolar core of the ER membrane in the translocon and moves laterally into the membrane and away from the translocon in a one-step process, driven by hydrophobic association of the TM sequence with

bilayer lipids and with little or no involvement of translocon proteins (Heinrich et al., 2000; Martoglio et al., 1995; Mothes et al., 1997).

Yet not all experimental data are consistent with this reasonable mechanism for integration. Three other crosslinking studies have observed that TM sequences do not move immediately into the bilayer, but are adjacent to translocon proteins for a prolonged period (Do et al., 1996: Meacock, 2002). These data suggest that TM sequences may not quickly diffuse away from the translocon, perhaps because of interactions between the TM sequence and translocon proteins.

To clarify the nature of TM sequence involvement with the translocon, if any, during cotranslational membrane protein integration, we have used a new experimental approach to ask directly whether the TM segment of a nascent membrane protein binds to a translocon protein(s). Our experiments reveal that during insertion, each TM segment is positioned adjacent to Sec61α in a specific orientation that is TM sequence-dependent and is maintained throughout integration, irrespective of TM segment location in the nascent chain or its orientation in the bilayer. The non-random TM segment interactions with and retention by the translocon can be effected only by protein-protein interactions between translocon proteins and each TM segment, not by nascent chain-phospholipid interactions. Our experiments therefore suggest a new paradigm for the mechanism of cotranslational TM sequence insertion into the ER membrane via the translocon, one in which the lateral movement of TM

segments through the translocon is actively regulated by protein-protein interactions between the translocon and the nascent chain.

The Nature of the TM-Translocon Interaction and the Role of the Flanking Regions

An important question that arises from the above results is what features of a TM segment are important for interaction with the translocon? To answer this question, we have focused on the nature of the protein-protein interactions between the integrating polypeptide and the translocon components, to clarify which regions of the nascent chain are involved in interactions with translocon proteins. By placing photoprobes at different positions in the TM sequence, we were able to map the points of interaction between the TM segment and the translocon. By replacing the flanking sequences of a TM segment, we were able to show that the important interactions regarding integration of the nascent protein occur primarily within the TM, not with the flanking regions. Furthermore, the primary sequence of the flanking region has a small role, if any, in the lateral movement of the TM segment into the membrane.

Multi-Spanning Membrane Protein Insertion

In the case of polytopic membrane proteins, the question of integration becomes more complex. The same simultaneous requirements of permeability and integration must be met, but within the context of moving multiple hydrophobic stretches laterally into the lipid bilayer. It is known that by alternating type I and type II TM domains, a desired final topology can be

reached (Wessels and Spiess, 1988); (Lipp and Dobberstein, 1988). (The C-terminal ends of Type II signal-anchor sequences are on the lumenal surface of the ER membrane, whereas the C-terminal ends of Type I stop-transfer sequences are located at the cytoplasmic surface of the ER membrane.) It is also known that the translocon and ribosome, in conjunction with the lumenal chaperone, BiP, are dynamic enough to allow for alternately translocating or releasing a loop into the lumen or cytosol, respectively, while still maintaining the permeability barrier. In a study using nascent chain fluorophores and their accessibility to quenching ions, Liao et al., (1997) observed that the ribosome●translocon seal is broken and BIP directly or indirectly seals the translocon pore from the lumenal side when the cytoplasmic portion of a single-spanning membrane protein needs to be released into the cytosol (Fig. 2). Upon translation of the subsequent TM, the ribosome presumably re-establishes the translocon seal and the following loop of the nascent chain is directed into the lumen. However, this has not yet been shown experimentally. In the case of a multi-spanning membrane protein, this process must repeat itself so that subsequent cytoplasmic and lumenal loops of the membrane protein can be properly localized.

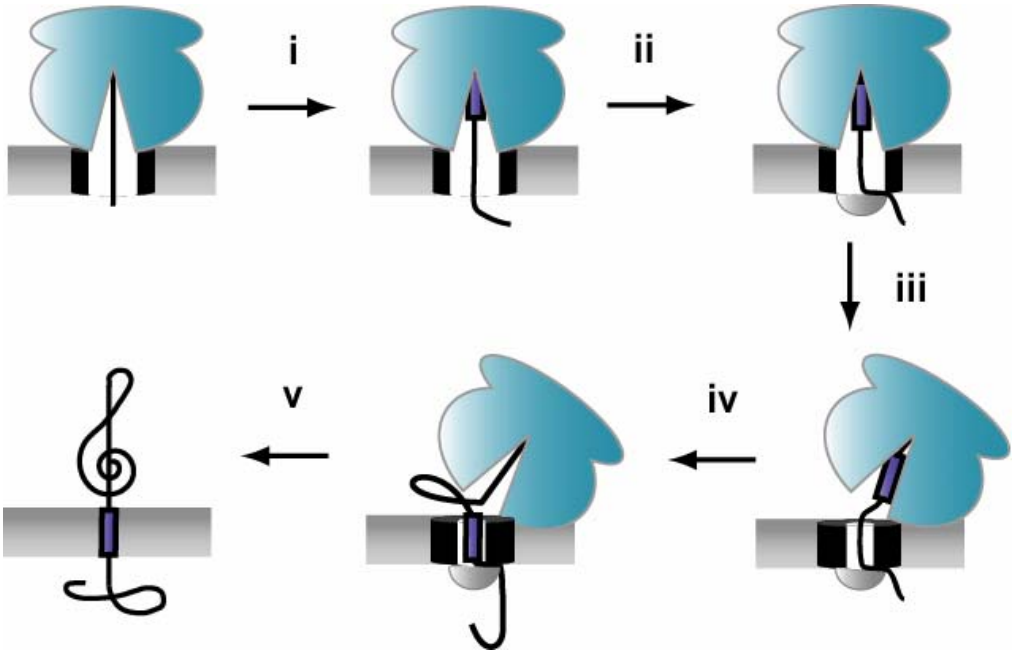

Figure 2 Nascent Chain Exposure during Membrane Protein Integration.

Immediately after the TM segment is synthesized (i), the system is in its translocation mode, with the lumenal end of the pore open and the ribosome binding to the translocon forming a tight seal. The TM segment is then detected inside the ribosomal tunnel when the C-terminal end of the TM segment is ~4 residues from the petidyl transferase center, the lumenal side of the pore is then sealed by the lumenal chaperone BiP. After five more residues are synthesized the ribosome-translocon seal is broken (iii), allowing the cytoplasmic domain of the nascent membrane protein to move into the cytoplasm (iv). For a signal-sequence cleaved single-spanning membrane protein the ribosome-translocon junction remains open until translation terminates (v), while BiP maintains the permeability by binding at the lumenal side. (Figure and Legend adapted from Johnson and Van Waes, 1999).

The active involvement of the translocon in TM insertion with single-spanning membrane proteins raises the question about translocon involvement and rentention of TM segments in the translocon during the integration of a multi-spanning membrane protein. One can use the same experimental approach to examine this issue, as well as to directly address the sequence of TM segment entry into and exit from the translocon.

Our studies of single-spanning membrane proteins revealed that each TM is recognized and held by the translocon (Chapter III). In that study we also found that the second TM sequence in a nascent chain was also bound and held by the translocon. This result was important for two reasons. First, it showed that TM segments in both orientations were recognized and held by the translocon. Second, it demonstrated that the translocon was actively engaged in the insertion of more than the first TM segment in a multi-spanning membrane protein. However, what was not examined was the timing of the interactions between TM1, TM2, and the translocon. Specifically, how are multiple TM sequences in the same polypeptide integrated into the bilayer?

Historically, this question has been examined using indirect methods. It was first proposed that polytopic membrane proteins were inserted using a 'linear' model in which the topology of the first TM segment determined the topology of all successive hydrophobic TM stretches (Blobel, 1980). It was then shown that one can indeed create a protein of predicted topology by alternating type II signal-anchor and type I stop-transfer TM sequences (Lipp and

Dobberstein 1988). However, in more recent investigations, it is clear that polytopic membrane protein insertion is more complicated than the linear model predicted. In the case of P-glycoprotein, Skach and Lingappa (1993), have shown that TM1 and TM2 can not independently integrate the polypeptide into the membrane. The two TM segments must act cooperatively to properly integrate the nascent chain.

The linear model was an attempt to understand and predict a protein's final topography. It did not address how the TM segments of multi-spanning membrane proteins are moved through the translocon and into the lipid bilayer. For example, the linear model does not address whether TM sequences are inserted one at a time, pair-wise, or all at once. To date, there are only two studies to have addressed this issue.

One study assessed TM insertion into the ER membrane by its sensitivity to extraction from the bilayer with urea. Any nascent chains that had inserted into the nonpolar core of the bilayer would presumably not be extracted from the membrane using either urea or high pH (carbonate extraction), while nascent chains that could be extracted from the membrane in urea were assumed to not have had any TM segments inserted into the bilayer. Using integration intermediates with different lengths of nascent chains and treating with 4.5 M urea, Borel and Simon (1996) concluded that the polytopic ABC-transporter protein P-glycoprotein, p-gp, was held within an aqueous-accessible compartment (presumably the translocon pore) until translation was complete,

at which point it was released into the bilayer. However, this approach does not directly determine individual TM segment insertion into the bilayer. More importantly, the indirect approach of Borel and Simon only measures the overall partitioning of the nascent chain between the aqueous and lipid phases, and hence is sensitive to the overall solubility of the nascent chain. It is therefore revealing that in their experiments, every nascent chain attached to a tRNA molecule was found in the aqueous phase, while every nascent chain released from the tRNA by puromycin was found in the membrane pellet. Hence, their conclusions about nascent chain insertion into the lipid bilayer may have been compromised by their experimental design and the high solubility of tRNA in water.

To date, only one study has focused on where a given TM segment is located in relation to another TM within the same polypeptide vis a vis the translocon (Heinrich and Rapoport, 2003). This study, focused on a bitopic membrane protein derived from the bacterial leader peptidase. Using integration intermediates with different lengths of nascent chain and a combination of chemical and photocrosslinking the authors observed that TM1 did not leave the translocon when TM2 emerged from the ribosomal tunnel. Instead, the authors show that a reduction in efficiency of crosslinking from TM1 of nearly 80% (from 6% to 1%) as TM2 emerges. Once TM2 enters the translocon, TM1 crosslinking increases to 3% only to taper off. From these results the authors concluded that the TM1 does not leave the vicinity of the

translocon and waits to interact with TM2 so that the segments may then insert in a cooperative pair wise manner. In addition, the authors report that when they replaced the native TM2 with an alternative TM segment from the asialoglycoprotein, TM1 moved away from the translocon as TM2 emerged from the ribosome. However, the raw data was not displayed. Heinrich and Rapoport conclude that the native TM1 and TM2 of leader peptidase interact and insert cooperatively, whereas replacing TM2 leads to independent parititioning of TM1 into the membrane. The raw data published (Heinrich and Rapoport, 2003) appear to suffer from aggregation problems, an effect that can significantly decrease crosslinking efficiency. The aggregation often occurs during the preparation of the sample for electrophoresis. While this would explain the much lower crosslinking efficiency that they observed between Sec61α and leader peptidase, it does not explain why they observe the co-emergence of TM1 and TM2, a result not seen in my experiments. It remains to be seen whether the discrepancy between the results of the two groups is explained by the difference in the constructs used or the procedures used.

We have expanded the approach of Do et al. (1996) to include multiple iterations of various polytopic membrane proteins with probes in different TM sequences and have investigated integration at a higher resolution than previously reported. Using this approach, we have determined that the incorporation of multiple TM segments is a hybrid of a sequential process and a pair-wise mechanism.

CHAPTER II

MATERIALS AND METHODS

Plasmids and mRNA

Construction of the plasmid encoding 111p has been described previously (Do et al., 1996; Johnson et al., 1995).

The plasmid 111p+O2p was created by digesting 352p (Chapter V) with *Apa*I and *Xba*I. The large fragment was purified from a 1.0% agarose gel using Qiagen's gel extraction kit. To create the insert, p211 (Chapter V) was digested with *Apa*I and *Xba*I and the small fragment was similarly purified using an agarose gel. The insert was then ligated into the vector using T4 DNA ligase (overnight, room temperature). XL1 blue cells were then transformed using heat shock (42°C, 1.5 min) and the DNA plasmids isolated using the Qiagen mini-prep kit. Plasmids were screened using an *Apa*I and *Xba*I double-digestion and positives were sequenced at the Gene Technologies Laboratory (Dept. Biology, TAMU). Lysine positions were moved within the O2 TM segment using overlap PCR mutagenesis with the following primers:

AJ135 (5'-GAAATGTTCAACGAACTCG-3')

AJ136 (5'-ATGGAGCCAGAGAAAGCA-3')

AJ201 (5'-GGCCGACAAATTCATGGTC-3')

AJ202 (5'-GACCATGAATTTGTCGGCC-3')

AJ203 (5'-CGACCTCAAGATGGTCTTC-3')

AJ204 (5'-GAAGACCATCTTGAGGTCG-3').

Plasmids containing the coding sequences for opsin, transferrin receptor (Tfr), and leader peptidase (Lep) were generously provided by Drs. Reid Gilmore, Carol Enns, and IngMarie Nilsson, respectively.

Lysines and amber codons were introduced into the parent plasmids at various positions within the transmembrane regions using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

The plasmids 111/O3p, 111/O5p, 352p, 211L41p, and 353p were made in the lab by Drs. Jialing Lin and Mingang Chen. A lysine codon was engineered into amino acid positions 79-82 in 111/O3p and positions 72-75 and 77-79 in 111/O5p. The leucine at position 76 was replaced with a proline in 111/O5p to create 111/O5p(P76L).

The plasmid Lep1 was mutated to introduce a single amber stop codon at amino acid positions 25-27 within the signal-anchor region using QuikChange by Stratagene. Tfr had probes introduced within its signal-anchor at positions 44-47. All Tfr mutations and experiments were performed in the lab by Yiwei Miao.

The plasmid 111/Lep1p was created by placing the signal-anchor of Lep1 in place of the VSV G TM in 111p. This was done using overlap PCR mutagenesis.

Truncated mRNAs coding for nascent chains of defined lengths were transcribed in vitro using SP6 RNA polymerase as before (Do et al., 1996) with either DNA cleaved by restriction endonucleases or PCR-produced DNA

fragments of the desired length. When endonuclease cleavage left a 3' overhang, the DNA was blunt-ended by synthesis (15 min, 22°C) with Klenow DNA polymerase prior to transcription.

tRNA

Yeast Lys-tRNA^{Lys} and εANB/TDB/BP -Lys-tRNA^{Lys} were purified and prepared as detailed earlier (Crowley et al., 1993; Krieg et al., 1986). *E. coli* tRNA^{Lys} and a derivative with a single base change in the anticodon that converted the tRNA^{Lys} into a tRNA that recognizes the amber stop codon (a generous gift of Dr. Greg Beckler, Promega Corp., here termed tRNA^{amb}) were synthesized in vitro using T7 RNA polymerase, aminoacylated, and modified as described elsewhere (Flanagan et al., 2003)

Translation and Photolysis

In vitro translations (26°C, 40 min, 50 µl) were performed in wheat germ cell-free extract as before (Do et al., 1996; Liao et al., 1997) in the presence of 40 nM canine SRP, 8 equivalents of column-washed rough microsomes (Walter and Blobel, 1983), 10 µCi of [^{35}S]Met, 32 pmoles of εANB/TDB/BP-Lys-tRNA$^{Lys/amb}$ as indicated, and other components as described (Crowley et al., 1993). Samples were photolyzed on ice for 15 min using a 500W mercury arc lamp. After photolysis, samples were sedimented through a 130 µl sucrose cushion [0.5 M sucrose, 25 mM HEPES (pH 7.5), 130 mM KOAc, 3 mM $Mg(OAc)_2$] in a Beckman airfuge (4°C, 5 min, 20 psi).

Immunoprecipitation of Single-Spanning Membrane Proteins

Microsome pellets were resuspended in 50 µl of 3% (w/v) SDS, 50 mM Tris-HCl (pH 7.5) and placed at 55°C for a minimum of 30 min. The volume was adjusted to 500 µl with buffer A [150 mM NaCl, 50 mM Tris-HCl (pH 7.6), 2% (v/v) Triton X-100, 0.2% (w/v) SDS] for TRAM-specific antibodies or buffer B [140 mM NaCl, 10 mM Tris-HCl (pH 7.6), 2% (v/v) Triton X-100] for Sec61α-specific antibodies. Samples were then pre-cleared by rocking with 30 µl of protein A-sepharose (Sigma) at 22°C for 1 hr before the Sepharose beads were removed by sedimentation. Sec61α- or TRAM-specific antiserum was added to each supernatant, and the samples were rocked overnight at 4°C, after which protein A-Sepharose (50 µl) was added to each sample and incubated for a minimum of 2 hr at 4°C. The immunoprecipitate was recovered by sedimentation, washed twice with buffer A or B, and then washed a final time with the same buffer containing no detergent. Immunoprecipitated material was then separated by SDS-PAGE as before (Do et al., 1996) and visualized using a Bio-Rad FX or GS-250 phosphorimager. Affinity-purified rabbit antisera to the C-terminal 14 and 13 amino acids of canine Sec61α and TRAM, respectively, were obtained from Research Genetics (Huntsville, AL).

Immunoprecipitation of Multi-Spanning Membrane Proteins

The conditions for the multi-spanning membrane proteins were adjusted to avoid aggregation of the more hydrophobic nascent chains. First, the resuspension temperature was decreased from 55°C to room temperature as

the higher temperature appeared to favor aggregation. Second, the detergent conditions were adjusted with the following detergents: SDS, Triton X-100, NP-40, CHAPS, and n-Octyl-β-D-glucopyranoside all tested in immunoprecipitation reactions. The pairing of SDS and Triton X-100 appeared to provide the best combination of detergent conditions. The concentrations of SDS and Triton X-100 were then optimized to provide the following conditions. In addition, it was found that increasing the ionic strength of the resuspension buffer aided protein recovery using immunoprecipitation. After optimization, microsome pellets were resuspended in 50 µl of 2% (w/v) SDS, 80 mM NaCl, 50 mM Tris-HCl (pH 7.5) and left at room temperature for a minimum of 30 min. The samples were then treated the same as the single-spanning membrane proteins except the protein A-sepharose was resuspended in a minimum of 50 µl of sample buffer. It was found that resuspension in a lower volume of sample buffer led to more aggregation of the multi-spanning membrane proteins.

Detection of Phospholipids in Photoadducts

Membrane pellets were resuspended in 50 µl of 5 mM $CaCl_2$, 100 mM Tris-HCl (pH 7.5), 1.0% (v/v) Triton X-100, and incubated at room temperature for 30 min before being brought to 200 µl with the same buffer. Each sample was split equally between two tubes and 2 units of phospholipase A2 (PLA_2) were added to one tube. After both tubes were incubated at 42°C for 30 min, 2 units of PLA_2 were added to the tube that lacked PLA_2 and both samples were

immediately precipitated in 12.5% (w/v) trichloroacetic acid. Samples were analyzed by 7.5%-17.5% SDS-PAGE.

CHAPTER III

TM INTEGRATION

Experimental Design

Bound vs. Free TM Segments

The primary issue under consideration is whether or not during integration a translocon protein binds to a TM segment after it enters the translocon and long after it would be free to diffuse away from the translocon. If it does not, the TM segment would move into the fluid bilayer, surrounded by phospholipid molecules that are too small, mobile, and flexible to restrict the local motion of the TM segment (Fig. 3A). Such a TM segment would be free to rotate and randomize its orientation relative to the nearby translocon. The translational diffusion of a TM segment would be restricted only by the length of the nascent chain tethering the TM segment to the ribosome-bound tRNA. In contrast, if the TM segment binds to a translocon protein(s), the position of the TM segment within the translocon would be fixed relative to the translocon proteins. The TM segment would be held at the binding site and would not be free to rotate or randomize its position relative to this binding site and translocon protein (Fig. 3B). These two possibilities can be distinguished by whether or not the positioning of the TM segment at the translocon is random.

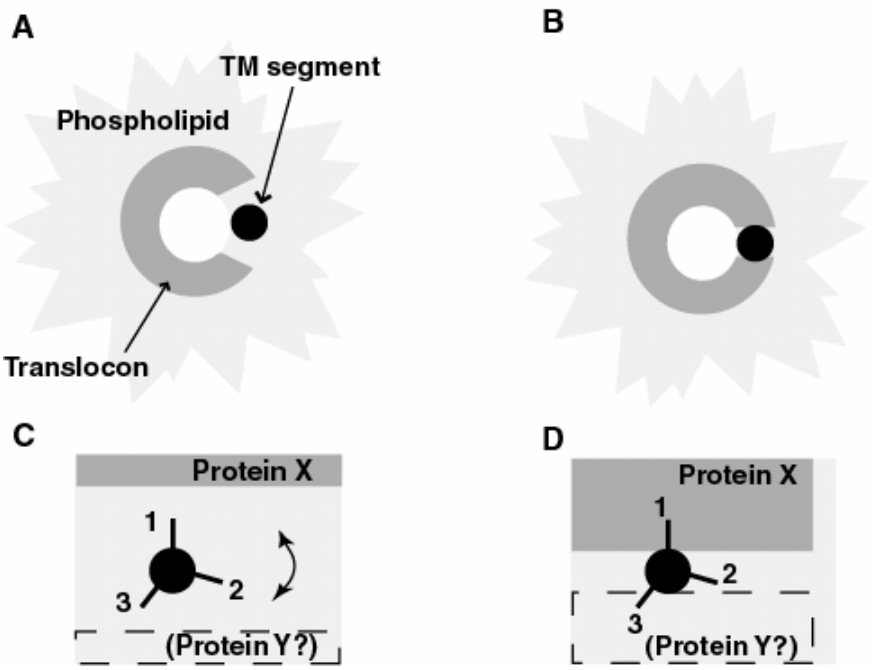

Figure 3 Two Possibilities for the Integration of a TM Segment into the ER Membrane.

In panels A-D, the translocon is viewed from the lumenal side of the ER membrane, and the TM segment is shown end-on as a black circle. (A) One model for the mechanism of TM segment movement from the aqueous pore into the lipid bilayer proposes that the TM sequence moves rapidly into the nonpolar core of the bilayer, with at most a transient interaction with translocon proteins. The TM segment is therefore surrounded by phospholipids very soon after reaching the translocon. (B) In another model, the nascent chain TM sequence binds to one or more translocon proteins after entering the translocon and is held until it is released laterally into the bilayer. (C) If the TM segment is surrounded by phospholipids and not bound to translocon proteins, then the TM segment will rotate and randomize its orientation relative to the translocon. Thus, no matter where a probe is initially located in the TM α-helix (e.g., extending in direction 1 or 2 or 3), the distribution of probes extending from the α-helix will be the same for all three samples at the time of photolysis. Thus, the fraction of probes that react with X (or Y) will be the same for all three original probe locations. (D) If a TM segment is bound to a translocon protein(s), then the TM α-helix will have a fixed position relative to that protein and will not be free to rotate. Thus, a probe attached to the TM segment will also have a fixed orientation relative to that protein. For example, if a photoreactive probe extends from the TM α-helix in direction 1, it would be expected to react covalently with translocon protein X upon photolysis. If the probe were instead substituted for the following residue in the TM α-helix, it would extend in direction 2 where it may or may not react covalently with protein X and/or another translocon protein Y (if there is a second protein nearby). Similarly, a probe two residues away from that pointing in direction 1 would extend in direction 3 on the other side of the TM α-helix where it would most likely react covalently with protein Y (if there) upon photolysis. The photocrosslinking yields and targets would therefore be predicted to differ for different probe locations in a translocon-bound TM α-helix.

The specificity of TM segment positioning can be addressed experimentally as follows. Assume that a TM segment α-helix is located in or near the translocon between two proteins (X and Y; the reasoning is the same if only X or Y is present), either bound to the proteins (Fig. 3D) or surrounded by phospholipid and not bound to the proteins (Fig. 3C). If one incorporates a photoreactive probe into the TM sequence at residue n, then the probe will extend from the surface of the TM α-helix at a certain point (arbitrarily indicated as “1” in Figs. 3C, D). If the TM segment is bound within the translocon and its position is fixed relative to X and Y (i.e., the TM segment is unable to rotate in the plane of the bilayer; cf. arrow in Fig. 3C), then the probe extending in direction 1 would be expected to react primarily with protein X when photolyzed (Fig. 3D). If instead the single probe is inserted at residue n + 1, then this probe will extend from the surface of the TM α-helix in a direction (“2”) that is rotated 100° around the helix axis from the position of “1.” Again, if the TM segment is bound within the translocon and hence fixed in position and unable to rotate, the probe extending in direction 2 may react with X and/or Y, or with neither, when photolyzed. Similarly, if a probe is located at residue n + 2, this probe will extend from the other side of the TM α-helix at an angle of 200° (“3”) from the direction of “1” (Fig. 3D). If the TM segment is bound and cannot rotate, one would expect to see the probe reactly solely or primarily with protein Y upon photolysis, as depicted in Fig. 3D. In short, if the TM segment containing a photoreactive probe is bound at the translocon and hence is fixed in position

and unable to rotate freely, then one would expect to see an asymmetric pattern of photoadduct formation in which the efficiencies of photocrosslinking to individual translocon proteins, and perhaps even the identities of the proteins, would vary with the position of the probe on the surface of the TM α-helix.

In contrast, if the TM segment is not bound to translocon proteins and is surrounded by phospholipids (Fig. 3C), then the α-helix will be able to rotate and its orientation relative to proteins X and Y will be randomized and not fixed. As a result, the probes in such a sample will be facing in all directions at the time of photolysis, no matter where the probe was originally placed in the TM segment. Furthermore, because of this randomization, the distribution of probe directions or orientations will be the same for all samples. As a result, the magnitude of nascent chain photocrosslinking to protein X (i.e., the fraction of probes directed towards X) will be the same for each original probe location (1, 2, or 3; Fig. 3C). Thus, if the TM segment is not bound to the translocon, the observed photocrosslinking targets and yields would be independent of the original location of the probe in the TM sequence.

One can therefore determine whether a TM segment is translocon-bound or is unbound and free by the asymmetry of the photocrosslinking yields and targets for three successive probe sites in the center of the TM α-helix. If the extents of photocrosslinking vary substantially and reproducibly, then the position of the TM segment is fixed and it is bound by a translocon protein. If the extents of photocrosslinking are very similar (a symmetric pattern), then the

TM segment is free to rotate and randomize its position relative to the translocon.

Integration Intermediates

Fully-assembled integration intermediates with nascent membrane protein chains of a defined length were prepared in vitro in the presence of ER microsomes and signal recognition particle (SRP) by translating mRNAs that were truncated in the coding region. Ribosomes halt when they reach the ends of such mRNAs, but they do not dissociate from the mRNA because the absence of a stop codon prevents normal termination from occurring. Thus, the nascent chain remains bound to the ribosome as a peptidyl-tRNA, and the length of the nascent polypeptide in the membrane-bound complex is dictated by the length of the truncated mRNA added to the translation. To examine the immediate environment of a TM segment at different stages of integration, we prepared integration intermediates with [^{35}S]Met-labeled nascent chains of different lengths, each with a single photoreactive probe located at or near the center of its TM segment.

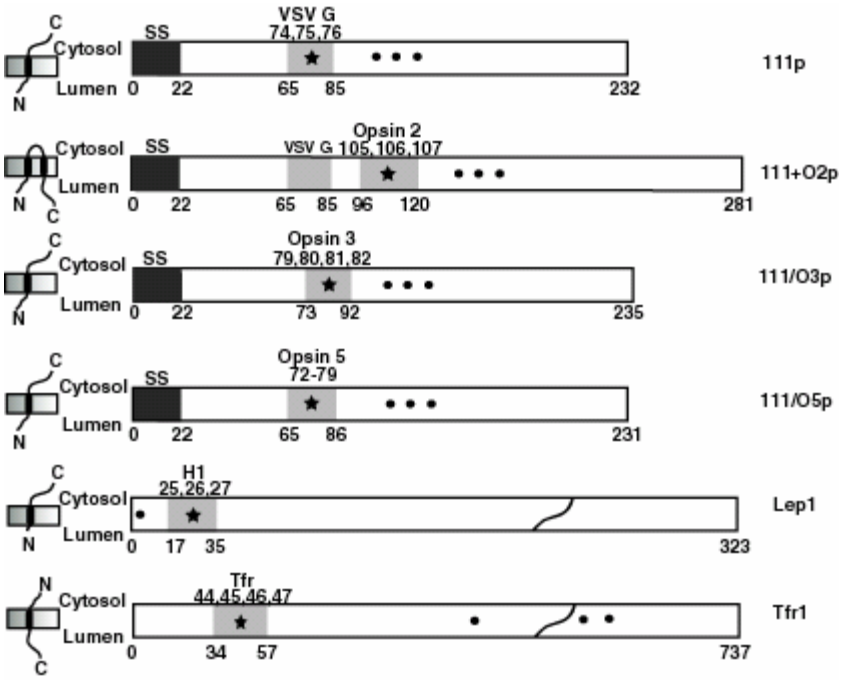

Figure 4 Membrane Proteins Used in This Chapter.

Several of the membrane proteins examined here have a preprolactin-derived signal sequence (SS) that is cleaved by signal peptidase, and a short stretch of invertase containing three glycosylation sites (circles) to indicate protein topography (this sequence is glycosylated only if it is on the lumenal side of the ER membrane) (Do et al., 1996). A single Lys or amber stop codon in each coding sequence directs the probe (star) to the indicated position in a TM segment. VSV G and TfR are the TM sequences of the VSV G and TfR proteins, respectively. H1 is the first TM sequence in leader peptidase, while the second, third, and fifth TM sequences in opsin are designated O2, O3 and O5.

The various membrane proteins examined in this study are shown in Fig. 4. Several proteins were constructed with a cleavable preprolactin (pPL) signal sequence at the N-terminus. These proteins were targeted to the ER membrane by SRP before the TM segment had been synthesized, so integration was separated from targeting in these experiments. For comparison, we also examined two membrane proteins termed signal-anchor proteins because their first topogenic sequence serves both as a signal sequence to target the nascent chains to the translocon and as a TM sequence to integrate and anchor the protein in the ER membrane. To specify the nascent chain used in an experiment, we have used the following nomenclature: $111p(75)_{93}$, where 111p identifies the protein, 75 indicates the location of the probe in this protein, and 93 gives the length of the nascent chain. Photoreactive probes were incorporated into nascent chains by translating the mRNA in the presence of N^{ε}-(5-azido-2-nitrobenzoyl)-Lys-$tRNA^{Lys}$ (εANB-Lys-$tRNA^{Lys}$) or εANB-Lys-$tRNA^{amb}$ that recognize either a lysine or an amber stop codon, respectively. To ensure that each nascent chain received only a single probe, the coding sequence of each protein used in this study contained either a single in-frame lysine codon or a single in-frame amber stop codon**.** It is important to note that each of these modified Lys-tRNAs incorporates an uncharged amino acid into the polypeptide instead of a charged lysine residue, so this experimental approach does not compromise the hydrophobicity of the TM segment.

Photocrosslinking to Sec61α

Previous studies have shown that the two primary protein targets for photoreactive probes in nascent membrane proteins are Sec61α and TRAM (Johnson and van Waes, 1999). Since SDS-PAGE analyses of the total photoadducts formed in the samples discussed below did not reveal any other major photocrosslinking targets (data not shown), the extent of nascent chain photocrosslinking to translocon proteins in each of the following experiments was assessed by immunoprecipitating the photolyzed samples with affinity-purified antibodies specific for either Sec61α or TRAM prior to analysis by SDS-PAGE.

When parallel samples of 111p(74)$_{112}$, 111p(75)$_{112}$, and 111p(76)$_{112}$ intermediates were photolyzed, immunoprecipitated with antibodies specific for Sec61α, and examined by SDS-PAGE, each of the [^{35}S]-labeled nascent chains was found to react covalently with Sec61α (Fig. 5). More importantly, the extent of photocrosslinking to Sec61α was very similar for probes positioned at positions 74, 75, or 76. Using the above rationale, it is clear that at this stage of the integration process, the center of the TM segment is exposed to Sec61α, but is not bound to a specific site in the translocon because the photocrosslinking efficiency was nearly the same for each probe location. Instead, the TM sequence appears to have just entered the translocon and is in the pore.

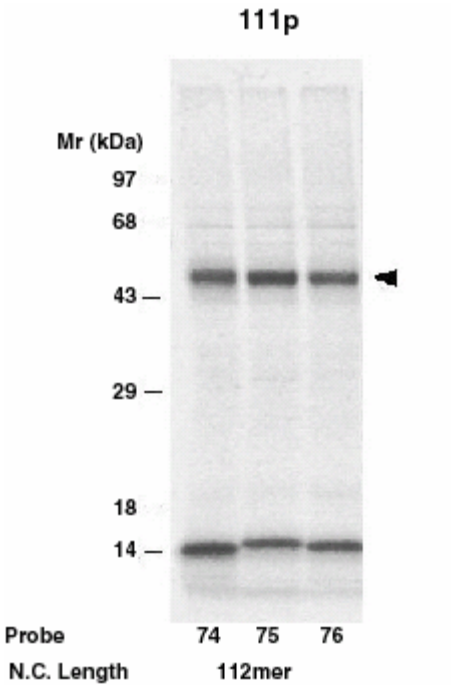

Figure 5 Photocrosslinking of 111p(74,75,76)$_{112}$ to Sec61α.

Integration intermediates were prepared in parallel using [^{35}S]Met, εANB-Lys-tRNA^{Lys}, and truncated mRNAs that yielded nascent chains of 111p(74), 111p(75), or 111p(76) with lengths of 112 residues. After photolysis, photoadducts containing Sec61α were purified by immunoprecipitation and SDS-PAGE, and are identified by the arrowheads. Molecular mass standards were electrophoresed to obtain the apparent molecular mass (Mr) values in kilodaltons (kDa).

However, a very different picture emerged when the system was examined at a later stage in the process (i.e., when the nascent chain was longer). When parallel samples of the three intermediates containing 154-residue nascent chains were photolyzed and analyzed, the extents of photocrosslinking to Sec61α differed markedly. A photoreactive probe located at residue 75 or 76 in the nascent chain reacted covalently with Sec61α, but a probe positioned at residue 74 did not photocrosslink to Sec61α (Fig. 6A). Thus, the side of the TM α-helix containing residues 75 and 76 faced Sec61α, while the other side faced away from Sec61α. Furthermore, since a probe at the end of the flexible lysine side-chain did not reach Sec61α from position 74 during the course of the photolysis, the TM segment was not free to change its orientation relative to Sec61α. The asymmetry of these photocrosslinking results therefore shows that the VSV G TM α-helix is positioned at a fixed site within the translocon in this integration intermediate.

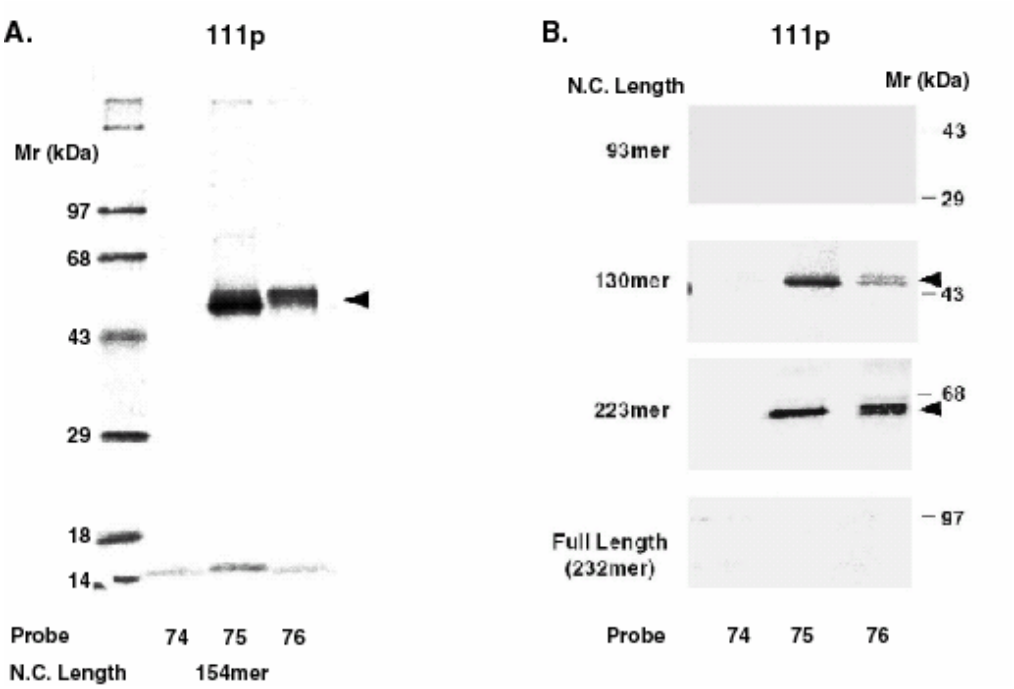

Figure 6 Photocrosslinking of 111p(74,75,76) to Sec61α at Longer Lengths.

Integration intermediates were prepared in parallel using [^{35}S]Met, εANB-Lys-tRNA^{Lys}, and truncated mRNAs that yielded nascent chains of 111p(74), 111p(75), or 111p(76) with lengths of (A) 154 residues, and (B) 93, 130, 223, and 232 (full-length) residues. After photolysis, photoadducts containing Sec61α were purified by immunoprecipitation and SDS-PAGE, and are identified by the arrowheads.

Does this asymmetric pattern of photoadduct formation persist throughout integration? Intermediates with nascent chains of different lengths were examined, and the results are summarized in Fig. 6B. When the nascent chain was short (93 residues) and the probe had not yet emerged from the ribosome, no photocrosslinks to Sec61α were observed. Similarly, when the full-length protein (232 residues) was translated, no photocrosslinking to Sec61α was observed. The photoreactive protein therefore diffused laterally away from the translocon after translation was completed. Yet photocrosslinking to Sec61α was observed when the nascent chain was long enough to allow the TM segment to enter the translocon (130 residues), and also when the nascent chain was very long and nearly full length (223 residues). In both of these cases, as well as for nascent chains of intermediate lengths (data not shown), the same general pattern of photocrosslinking was observed: no crosslinking from position 74, but crosslinking from positions 75 and 76. Thus, the same surface of the TM segment appears to face Sec61α throughout integration. It is also important to note that the extent of photocrosslinking to Sec61α did not change significantly as the length of the nascent chain increased, so the TM segment remained in the translocon until translation terminated.

Although the relative proximity of residues 74 and 75 to Sec61α does not change detectably during integration, the relationship of residue 76 to Sec61α does appear to alter over the course of integration. Whereas εANB-Lys photocrosslinking to Sec61α is much greater from position 75 than from position

76 when the nascent chain is 130 residues long, the extents of photocrosslinking from these positions are about the same when the nascent chain is 223 residues long (Fig. 6B). This change in the crosslinking pattern indicates that the TM segment may move relative to Sec61α during the integration process, but the magnitude of such movement cannot be determined from the extent of photoadduct formation.

We always observe a doublet when photocrosslinks between Sec61α and position 76 are examined (e.g., Fig. 6B). The most likely explanation for the appearance of two Sec61α photoadducts is that the probe at residue 76 reacts covalently with two different sites on Sec61α, and that the resulting photoadducts have different electrophoretic mobilities in SDS-PAGE. A precedent for such an occurrence has been described by Plath et al. (Plath et al., 1998).

As shown in Fig. 6, the VSV G protein TM sequence in 111p remained adjacent to the translocon long after the nascent chain tether was long enough to allow the TM segment to diffuse away from the translocon. In fact, the extent of photocrosslinking diminished only slightly even after more than 500 Å of fully-extended nascent polypeptide [(223 - 75) residues x 3.5 Å /residue] separated the probe in the TM segment from the tRNA in the ribosomal P site. Since the TM segment did not release from the translocon even with this long tether, the translocon appears to control both the location and the movement of the TM segment throughout the integration process.

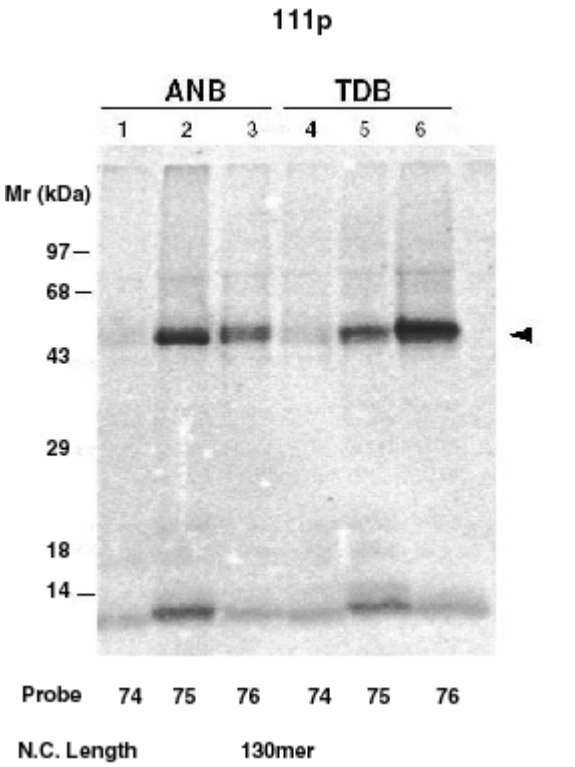

Figure 7 Examing the Effect of Probe Chemistry on Photocrosslinking.

Integration intermediates containing 130-residue nascent chains of 111p(74), 111p(75), or 111p(76) were prepared in parallel with either 32 pmol εANB-Lys-tRNA^{Lys} (ANB) or 32 pmol εTDB-Lys-tRNA^{Lys} (TDB), and were analyzed as above. The arrowhead identifies the photoadduct.

Photocrosslinking Asymmetry Is Independent of Probe Chemistry

It is conceivable that the absence of 111p(74)-Sec61α photocrosslinking results not from an absence of proximity, but rather from an inefficient chemical reaction. To examine this possibility, experiments were done with Lys-tRNAs modified with other photoreactive groups, either trifluoromethyldiazirinobenzoyl (TDB) or a benzophenone (BP) moiety. In one experiment, two samples of integration intermediates with $111p(74)_{130}$, $111p(75)_{130}$, or $111p(76)_{130}$ nascent chains were prepared in parallel. Each pair of samples was identical except for the photoprobe incorporated into the nascent chain: one received the nitrene-generating εANB-Lys and one received the carbene-generating εTDB-Lys. When the photolyzed samples were examined for crosslinking to Sec61α, it was clear that the carbene-containing nascent chains also reacted covalently with Sec61α (Fig. 7). Furthermore, the carbene- and nitrene-containing samples exhibited the same asymmetry in photocrosslinking: probes at residues 75 and 76 reacted with Sec61α, but probes at residue 74 did not. The same asymmetry in crosslinking was also observed with a third set of samples prepared with the benzophenone photoreagent (data not shown). Since the same asymmetry of nascent chain photocrosslinking to Sec61α was seen with three probes that have different excited state lifetimes and different reaction chemistries, and since each of the photoprobes reacted covalently with Sec61α with only slightly different efficiencies, the lack of photoadduct formation from position 74 is not likely due to probe chemistry, but rather to a lack of proximity to Sec61α. The

photocrosslinking data therefore accurately indicate which TM α-helix surfaces are facing or adjacent to Sec61α.

Photocrosslinking to TRAM

The exposure of TRAM to different surfaces of the VSV G TM α-helix during integration was examined using the same approach as detailed above for Sec61α. When parallel samples of integration intermediates containing 111p(74)$_{154}$, 111p(75)$_{154}$, or 111p(76)$_{154}$ were photolyzed and analyzed, photocrosslinking of the TM segment to TRAM was not equivalent from the three different sites on the TM α-helix (Fig. 8A). Probes located at positions 74 and 75 each reacted covalently with TRAM, but only a small amount of crosslinking to TRAM occurred from position 76. Thus, the TM segment is adjacent to TRAM in the translocon. Furthermore, since the extent of photocrosslinking to TRAM depends on the location of the probe in the TM segment, the TM segment occupies a fixed position in the translocon with respect to TRAM.

Integration intermediates with nascent chains of different lengths were also examined to determine whether the above photocrosslinking pattern extended throughout the integration process. None of the probes reacted covalently with TRAM either before the TM segment emerged from the ribosome (the 93-residue nascent chain in Fig. 8B) or after translation terminated and the full-length protein diffused away from the translocon (the 232mer in Fig. 8B). But for all nascent chains long enough to allow the TM segment into the translocon (130 and 223 residues, Fig. 8B; 171 residues, data not shown), the TM segment was photocrosslinked to TRAM. However, in each case TRAM was crosslinked much less efficiently from residue 76 than from either 74 or 75, thereby showing that the TM segment maintains its position during the stages of integration examined here.

Complementary Photocrosslinking to TRAM and Sec61α

Is differential photocrosslinking of nascent chains to TRAM and to Sec61α detectable in a single sample? Three samples of integration intermediates containing 111p(74)$_{154}$, 111p(75)$_{154}$, or 111p(76)$_{154}$ nascent chains were prepared in parallel and photolyzed. Each sample was then split and immunoprecipitated with antibodies specific for either Sec61α or TRAM. By comparing the extent of photocrosslinking to TRAM and to Sec61α in the same sample, one can determine directly whether probes at different locations in the TM segment react with different translocon proteins.

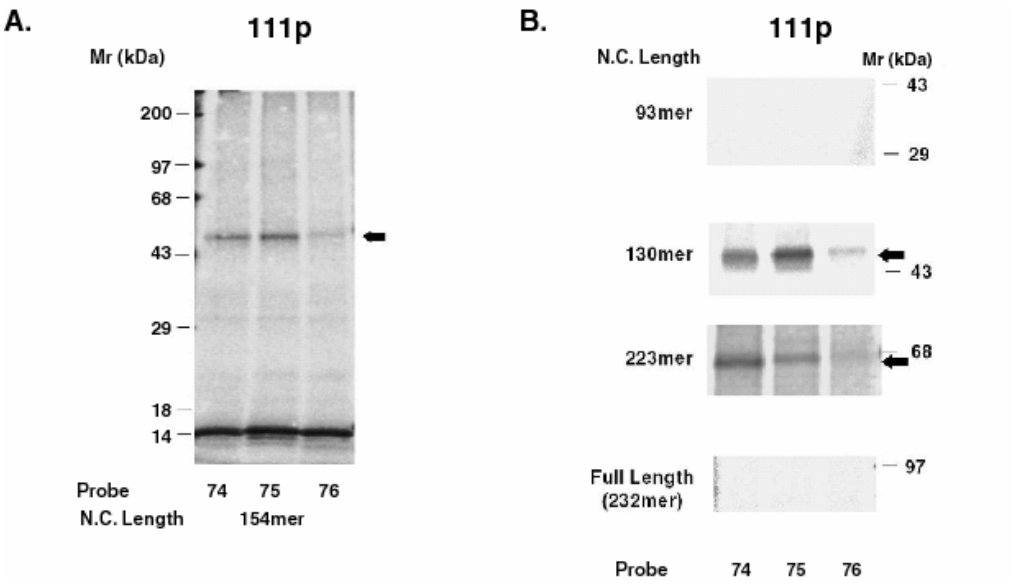

Figure 8 Photocrosslinking of Nascent 111p to TRAM.

Integration intermediates containing 111p(74), 111p(75), or 111p(76) nascent chains with lengths of (A) 154 residues or (B) 93, 130, 223, and 232 (full-length) residues were examined as in Fig. 5. After photolysis, photoadducts containing TRAM were purified by immunoprecipitation and SDS-PAGE, and are identified by the arrowhead.

By comparing lanes 1 and 4 in Fig. 9, it is clear that a photoreactive probe at residue 74 reacts covalently with TRAM, but not with Sec61α. In contrast, a probe at residue 76 reacts with Sec61α, but only poorly with TRAM (compare lanes 3 and 6, Fig. 9). Since residues 74 and 76 are located on opposite sides of the TM α-helix, it would appear that the VSV G TM segment in 111p is positioned between TRAM and Sec61α in the translocon, with residue 74 facing TRAM and residue 76 facing Sec61α. It is also clear that the TM segment is close enough to each protein to photocrosslink to it. Since a probe at residue 75 reacts covalently with both TRAM and Sec61α, it would appear that this probe is located between the proteins where it can contact each (see direction 2 in Fig. 3D). The fraction of 111p(75) nascent chains that crosslinked to translocon proteins averaged 15% for nascent chain lengths ranging from 130 to 230. Thus, the efficiency of photocrosslinking was high and did not decrease much as the nascent chain lengthened.

The Translocon also Binds Other TM Segments

The above experiments were done with a single-spanning membrane protein whose TM sequence is identical to that of the vesicular stomatitis virus G (VSV G) protein except for the probe residue. To determine whether other TM segments are bound by the translocon as they move laterally into the bilayer, other membrane proteins were prepared from the 111p chimera and examined. Two proteins were the same as 111p except that the VSV G TM sequence of 111p was replaced by either the third or the fifth TM sequence (O3

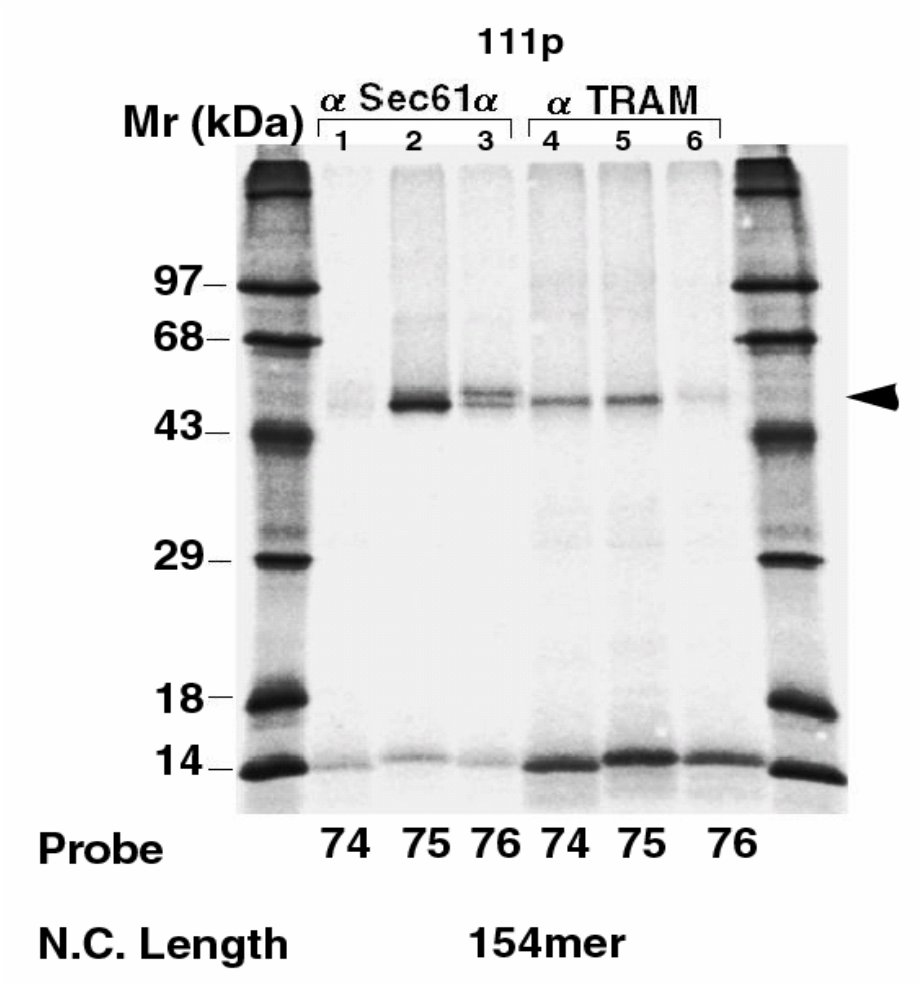

Figure 9 Direct Comparison of Photocrosslinking to Sec61α and TRAM in the Same Sample.

Integration intermediates containing 154-residue nascent chains of 111p(74), 111p(75), or 111p(76) were prepared as in Fig. 5 and photolyzed. After the microsomes were pelleted and resuspended, 60% of each sample was immunoprecipitated with antibodies to TRAM and the other 40% was immunoprecipitated with antibodies to Sec61α before analysis as in Fig. 5. Photoadducts containing Sec61α and TRAM are indicated by the arrowhead.

or O5) of bovine opsin to yield proteins designated 111/O3p and 111/O5p (Fig. 4). Derivatives of each protein were prepared, each with a single lysine codon positioned in place of one of the residues in the middle of O3 or O5. When translated in the presence of ER microsomes, SRP and either pre-flashed or DTT-inactivated εANB-[^{14}C]Lys-tRNA^{Lys}, each of these proteins was fully integrated into the ER membrane in the proper orientation, as determined by their insensitivity to extraction with 0.2 M sodium carbonate (pH 11.5) and the absence of glycosylation (data not shown). These TM segments were therefore integrated normally despite the presence of the probe. It is also important to note that each of these TM segments (VSV G, O3, and O5), as well as the other TM segments discussed below, has the same orientation in the ER membrane as in the native protein.

Integration intermediates with a 148-residue 111/O3p nascent chain containing a photoreactive probe at position 79, 80, 81, or 82 were photolyzed in parallel, and each nascent chain photocrosslinked to Sec61α (Fig. 10). However, the extent of photoadduct formation was very different for the four different probe locations in O3. Whereas a probe at position 79 in the nascent chain reacted relatively efficiently with Sec61α, little photocrosslinking to Sec61α was observed when the probe was located at position 80, and intermediate extents of photoadduct formation were observed when the probe was substituted for residue 81 or 82. Since similar differences in the extents of photocrosslinking to Sec61α were observed with longer nascent chains, O3

does not appear to move significantly within the translocon as integration proceeds. Furthermore, the total extent of 111/O3p(79) photocrosslinking to Sec61α (14% average) was approximately constant as the length of the nascent chain increased to more than 210 residues (data not shown), which shows that O3 is not released from the translocon soon after entering it. Since probes extending from different sides of the O3 α-helix do not have equal access to Sec61α, O3 is not oriented randomly with respect to Sec61α in the translocon. Thus, O3 must be bound within the translocon via protein-protein interactions, as was observed for the VSV G TM segment.

An asymmetry in the photocrosslinking of 150-residue 111/O5p nascent chains to Sec61α was observed when the photoprobe was placed at different locations within O5 (Fig. 11, where the crosslinking yield from position 79 was 16%). Thus, this TM segment is also bound within the translocon during integration. But since the pattern of photocrosslinking differs for VSV G, O3, and O5, the interactions of these TM segments with the translocon are not identical. This conclusion is further underscored by the discovery that little photocrosslinking of either 111/O3p or 111/O5p nascent chains to TRAM occurred from any of the probe locations. It therefore appears that the proximity of TM segments to TRAM varies during integration.

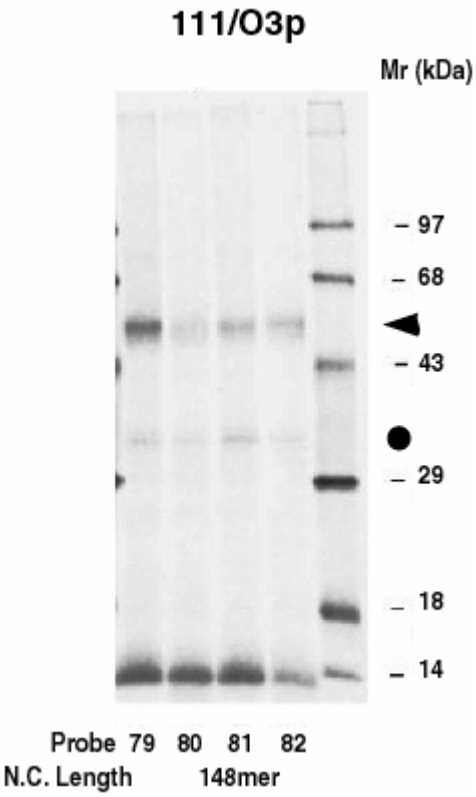

Figure 10 Photocrosslinking of 111/O3p to Sec61α.

Integration intermediates were prepared, photolyzed, and immunoprecipitated with antibodies to Sec61α as in Fig. 6. Samples contained 148-residue nascent chains of 111/O3p with a probe at residue 79, 80, 81, or 82. Photoadducts containing Sec61α are identified by the arrowhead. The weak radioactive bands identified by the black circle are not εANB-Lys photoadducts, but are residual peptidyl-tRNAs that contain the nascent chain.

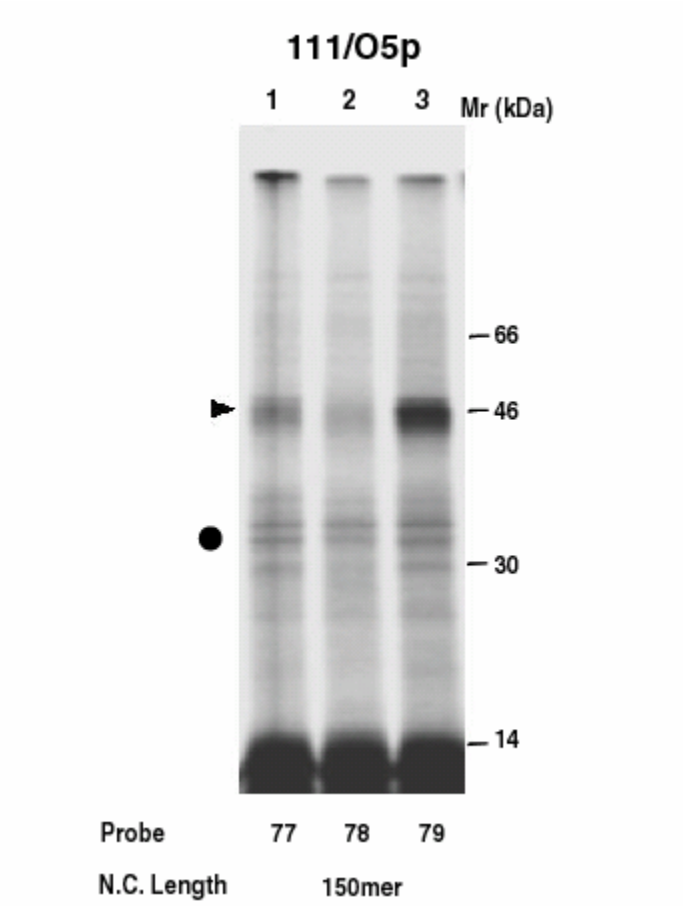

Figure 11 Photocrosslinking of 111/O5p to Sec61α.

Integration intermediates were prepared, photolyzed, and immunoprecipitated with antibodies to Sec61α as in Fig. 5. Samples contained 150-residue nascent chains of 111/O5p with a probe at residue 77, 78, or 79. Photoadducts containing Sec61α are identified by the arrowhead. The weak radioactive bands identified by the black circle are not εANB-Lys photoadducts, but are residual peptidyl-tRNAs that contain the nascent chain.

The Translocon also Binds TM Segments in a Multi-Spanning Membrane Protein, Including Those with Opposite Orientations in the Bilayer

To examine translocon interactions with a TM segment that is both oriented in the opposite direction in the bilayer from the above TM sequences and is also the second TM sequence in the nascent chain, the second TM sequence of bovine opsin (O2) was inserted into 111p behind the VSV G TM sequence to yield 111+O2p (Fig. 4). Three derivatives of this double-spanning membrane protein were prepared by replacing residue 105, 106, or 107 with a Lys codon in the middle of TM2. When translated in the presence of ER microsomes and SRP, each of these proteins was integrated into the ER membrane in the proper orientation, as determined by carbonate extraction, protease protection, and glycosylation assays (data not shown).

When photolyzed, intermediates containing 167- or 178-residue 111+O2p nascent chains reacted covalently with Sec61α with high, intermediate, and very low efficiency when the photoreactive probe was at position 105, 107, or 106, respectively (Fig. 12). In addition, the Sec61α photoadducts appear to have different electrophoretic mobilities in the gel (compare lanes 5 and 7, and 1 and 3 in Fig. 12), which suggests that probes located at positions 105 and 107 crosslink to different sites on Sec61α (cf. Plath et al., 1998). As is true for all of the photoadducts discussed in this paper, photocrosslinking requires the photoreactive probe because samples that

incorporate unmodified Lys instead of εANB-Lys are unable to photocrosslink to Sec61α (compare lanes 4 and 5 in Fig. 12), even though both nascent chains are integrated and oriented properly. The great disparity in the extents of photocrosslinking to Sec61α from different sides of the O2 α-helix demonstrate that the position of O2 relative to Sec61α is fixed and is not random. Thus, the second transmembrane domain in a membrane protein is also bound during the integration process, even though its orientation within the bilayer is opposite to that of the above TM segments.

Photocrosslinking Is TM Sequence-Dependent

Is the location of a TM sequence in the translocon determined by the binding of the flanking sequences to the ribosome, translocon, and/or lumenal proteins, or by the binding of the TM sequence to a translocon protein(s)? If the non-random positioning of the TM sequence in the translocon results from the binding of the TM segment to a translocon protein(s), a single amino acid change at the binding site may alter the TM helix surface sufficiently to create a different binding site and photocrosslinking pattern. To test this possibility, we replaced the naturally-occurring Pro in the middle of O5 with Leu. Such a change could conceivably alter the conformation of a TM helix, but Wigley et al., (2002) showed that a disproportionate number of Pro residues are located in TM sequences and that the presence of Pro enhances α-helix formation by TM peptides in detergent micelles. Since the O5 α-helix is not kinked or distorted in

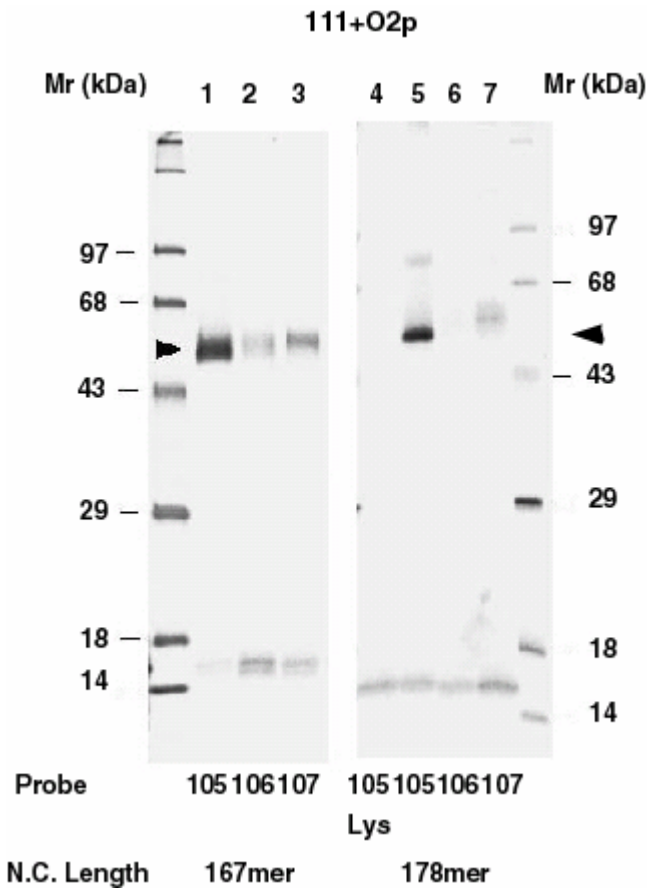

Figure 12 Photocrosslinking of 111+O2p to Sec61α.

Integration intermediates were prepared, photolyzed, and immunoprecipitated with antibodies to Sec61α as in Fig. 5. Samples contained 167- or 178-residue nascent chains of 111+O2p with a probe at residue 105, 106, or 107. The samples in lane 4 received unmodified Lys-tRNA instead of εANB-Lys-tRNA. Photoadducts containing Sec61α are identified by the arrowhead.

the rhodopsin crystal structure (Palczewski et al., 2000) replacing the Pro with Leu would not be expected to significantly alter the conformation of the helix. However, the van der Waals surfaces of the Pro and Leu are very different and would yield substantially different helical surfaces.

When integration intermediates containing 160-residue 111/O5p nascent chains with either the wild-type Pro or the P76L mutation were prepared and photolyzed in parallel, the extents of photocrosslinking of probes at positions 73 and 75 were substantially altered, from 0% to 15% (Fig. 13; compare lanes 2 and 4 with 6 and 8). Thus, a change in the amino acid sequence of the TM segment altered its orientation with respect to Sec61α, even though the flanking sequences were identical in the two proteins. This result is consistent with the idea that the TM sequence and the translocon engage in protein-protein contacts during integration.

These data also show clearly that the asymmetry in photocrosslinking is not due to differences in probe depth in the bilayer. The α-carbons in successive residues in a TM α-helix are displaced by 1.5 Å per residue perpendicular to the plane of the membrane (“vertically”). However, the probes are located at the end of a long lysine side-chain that places them about 15 Å from the α-carbons when fully extended. Since this side-chain is flexible, each probe can sample a large volume and there is substantial vertical overlap in the space accessible to probes on adjacent helical residues. Hence, a 1.5 Å

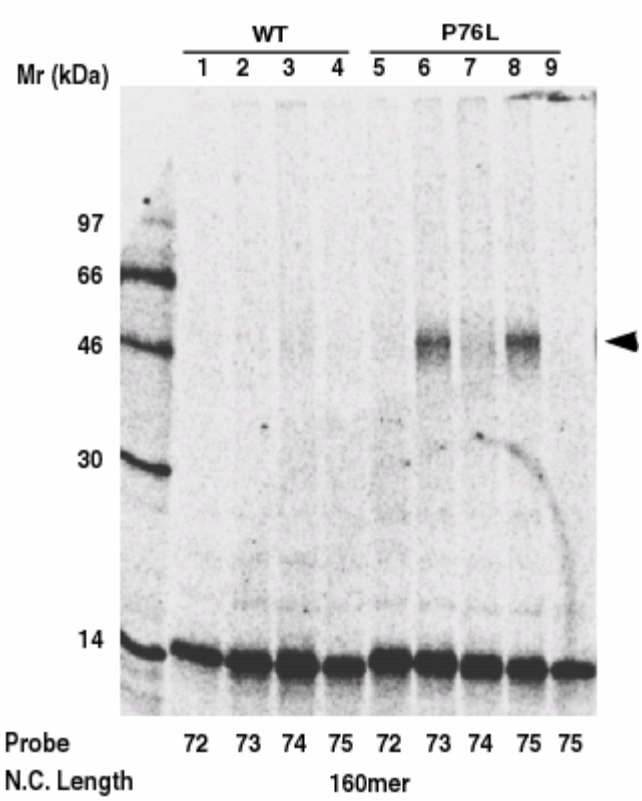

Figure 13 Photocrosslinking of 111/O5p P76L to Sec61α.

Integration intermediates were prepared, photolyzed, and immunoprecipitated with antibodies to Sec61α as in Fig. 6. Samples contained 160-residue nascent chains of 111/O5p (probes at residues 72, 73, 74 and 75 in lanes 1-4, respectively) or 111/O5p(P76L) (probes at residues 72, 73, 74 and 75 in lanes 5-8, respectively). The samples in lane 9 received unmodified Lys-tRNA instead of εANB-Lys-tRNA. Photoadducts containing Sec61α are identified by the arrowhead.

difference in the site of attachment to the helix is not likely to greatly affect the frequency with which a probe will contact a particular reactive site as the probe samples the volume available to it. In particular, adjacent probe locations would not change from zero to maximum and back to zero crosslinking (e.g., lanes 5-8, Fig. 13) because of a 1.5 Å difference in depth in the bilayer, simply because of the large overlap in space that is accessible to these probes.

The Translocon Binds to Signal-Anchor Sequences during Integration

To assess the generality of the above results, we examined two signal-anchor TM sequences that are naturally oriented in opposite directions in the ER membrane. Derivatives of leader peptidase (here designated Lep1) were prepared that contained a single amber stop codon at three successive locations within the signal-anchor sequence (Fig. 4). These constructs lacked the second TM sequence of leader peptidase and contained an N-terminal glycosylation site to facilitate topography determinations, similar to leader peptidase constructs used previously (Heinrich et al., 2000; Mothes et al., 1997). When Lep1 integration intermediates containing 140-residue nascent chains were irradiated, the probes at positions 26 and 27 photocrosslinked to Sec61α with high efficiency (15% of the total nascent chains were crosslinked to Sec61α via probes located 113 residues from the peptidyltransferase center); no photoadduct formation was observed when the probe was located at position 25 (Fig. 14A). The asymmetry of the photocrosslinking data indicates that the signal-anchor sequence is bound to the translocon.

Is a signal-anchor sequence released from the translocon more quickly than an internal TM sequence? After preparing integration intermediates with nascent chains of different lengths, we observed that the signal-anchor sequence was in proximity to Sec61α even in very long nascent chains (Fig. 14B). The extent of photoadduct formation decreases as the length of the nascent chain increases, but significant photocrosslinking was observed even when the length of fully-extended polypeptide between the probe in the signal-anchor domain and the tRNA in the ribosomal P site is between 396 Å (3.5 Å/residue x 113 residues) and 728 Å (3.5 x 208). This does not agree with the data of Heinrich et al. (2000), in which a maximum of ~15% photocrosslinking to Sec61α was reported to fall off sharply to ~1% when the TM probe was less than 58 residues from the peptidyltransferase center. The reason for this discrepancy is currently unclear. In our hands, the Lep signal-anchor domain does not immediately diffuse away from the translocon, but instead remains bound to the translocon for a substantial length of time.

We also examined the interactions of another signal-anchor sequence with the translocon during integration. The transferrin receptor (TfR) is a single-spanning membrane protein with a large hydrophilic N-terminal domain that ends up in the cytoplasm. For our study, we used a modified TfR that had much of the cytoplasmic domain deleted; this does not affect its processing either in vitro or in vivo (Rutledge et al., 1998). When integration intermediates containing carbonate-nonextractable and photoreactive 107-residue nascent

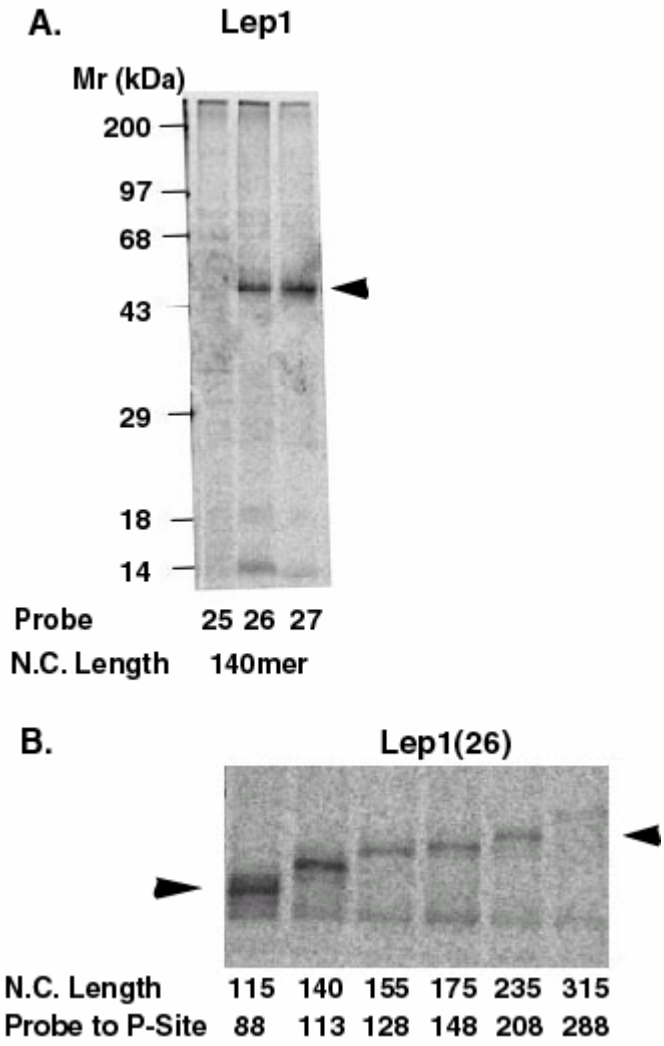

Figure 14 Photocrosslinking of the Signal-Anchor Protein Lep1 to Sec61α.

Integration intermediates were prepared using [^{35}S]Met, εANB-Lys-tRNA^{amb}, and truncated mRNAs that yielded nascent chains of: (A) Lep1(25), Lep1(26), and Lep1(27) at 140 residues and (B) Lep1(26) at the indicated lengths. After photolysis, photoadducts containing Sec61α were purified by immunoprecipitation and SDS-PAGE. The arrowhead identifies the photoadduct.

chains of the various TfR derivatives (Fig. 4) were photolyzed, the efficiency of photocrosslinking to Sec61α was found to differ depending upon the location of the probe in the TfR TM sequence (Fig. 15). This asymmetry indicates that both signal-anchor sequences and internal TM sequences, in either orientation, are bound to a translocon protein(s) during integration.

The TM Segment Is Adjacent to Phospholipids during Integration

To assess TM segment exposure to phospholipids during insertion, we prepared integration intermediates with a carbene photoreagent (TDB) and used the same procedures reported by Martoglio et al. (1995) and Mothes et al. (1997) to detect nascent chain photocrosslinking to phospholipids. When integration intermediates containing photoreactive 150-residue nascent chains of either 111p(75) or Lep1(26) were photolyzed, 38% and 48%, respectively, of the nascent chains were photocrosslinked to phospholipid molecules (Fig. 16). Thus, the TM sequences were adjacent to both translocon proteins and to phospholipids during integration. Since the nascent chain probes reacted 2-3 times more frequently with a phospholipid molecule than with a translocon protein at these nascent chain lengths, the probe in the TM segment is either adjacent to more phospholipid molecules than proteins at the time of photolysis and/or the covalent reaction proceeds more efficiently with phospholipids than with proteins. But the most important point is that the TM sequence is adjacent to both phospholipids and translocon proteins in the translocon when the nascent chain is still bound to translocon proteins (Figs. 6, 10, 11, 12, 14).

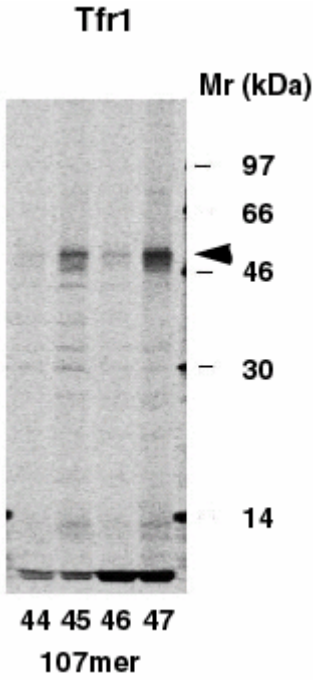

Figure 15 Photocrosslinking of the Signal-Anchor Protein Tfr to Sec61α.

Integration intermediates were prepared using [^{35}S]Met, εANB-Lys-tRNA^{amb}, as in Fig. 13. Samples contained nascent chains of Tfr at 107 residues with a probe at residue 44, 45, 46, or 47. After photolysis, photoadducts containing Sec61α were purified by immunoprecipitation and SDS-PAGE. The arrowhead identifies the photoadduct.

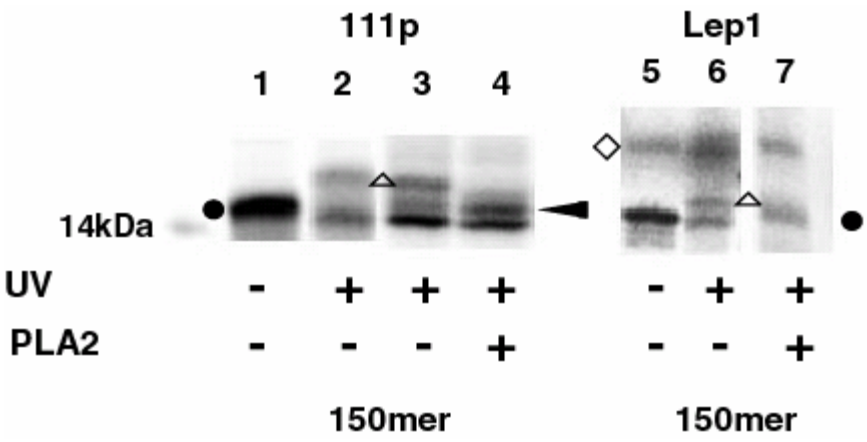

Figure 16 Photocrosslinking to Phospholipids.

Integration intermediates were prepared using [^{35}S]Met, εTDB-Lys-tRNA^{amb}, and truncated mRNAs that yielded either 150-residue 111p(75) or 150-residue Lep1(26) nascent chains. After photolysis, photoadducts containing phospholipids were identified using phospholipase A2 (PLA2). The sample in lane 3 received a mock PLA2 treatment. Unreacted nascent chains are indicated with a closed circle, the phospholipid-nascent chain photoadduct with an open triangle, the photoadduct after treatment with PLA2 with an arrowhead, and the glycosylated form of Lep1 with the open diamond.

Discussion

The experimental data presented in this chapter indicate that protein-protein interactions exist between the nascent chain and the translocon during cotranslational membrane protein integration. The data therefore support a major shift in paradigm, from the current model of little or no translocon protein involvement during TM sequence insertion into the ER membrane to a model in which protein-protein interactions mediate insertion and release from the translocon.

The environment of a nascent membrane protein during its integration into the ER membrane was examined by placing a photoreactive probe in the middle of a TM segment. The molecules adjacent to the TM segment were then identified at different stages of the integration process (i.e., at different lengths of nascent chain) by photocrosslinking. Probes placed at different locations around each of seven different TM α-helices reacted covalently with Sec61α and TRAM to different extents, thereby showing that the different surfaces of each TM segment were exposed to different protein environments within the translocon. Since the extent of photoadduct formation varied for each probe location within each TM segment, the TM segments were not located randomly within or alongside the translocon. Instead, each TM segment was reproducibly located in a specific position relative to Sec61α and TRAM at each stage of integration. Such a fixed positioning could only be achieved if the motion of the TM segment in the translocon were restricted to prevent its rotation and

translation relative to the translocon proteins, and this in turn could only be accomplished if the nascent chain were bound to the translocon. If the TM segment were instead surrounded by and interacted solely with phospholipids, the small, flexible, and mobile phospholipids would not be able to prevent the TM segment from rotating around a helix axis perpendicular to the plane of the membrane and thereby randomizing its orientation relative to nearby translocon proteins prior to photolysis, especially for long nascent chains. In such a situation, the extent of photocrosslinking would be the same for each probe location on the α-helical surface of a TM segment (see Fig. 5) because the final probe distribution (i.e., what fraction would be facing a particular direction or translocon protein surface) would be the same no matter where the probe was originally placed.

Each of the seven different TM sequences we have examined so far photocrosslinks asymmetrically to Sec61α. Thus, the translocon appears to bind to a stretch of nascent chain recognized as a TM sequence, irrespective of its orientation in the bilayer, its position in the nascent chain (first, second, or third topogenic sequence), or whether it is a signal-anchor sequence or an internal TM sequence. Furthermore, each TM sequence was retained within the translocon for an extended period of time, long after the nascent chain length was sufficient to allow the TM segment to diffuse away from the translocon and into the bilayer. The simplest explanation for these observations is that a

translocon protein(s) binds to a nascent chain TM sequence during its passage through the translocon into the ER membrane.

The transient association of a nascent chain TM sequence with a translocon protein TM sequence(s) during integration most likely involves primarily van der Waals interactions, since a hydrogen bond or a salt bridge between two TM sequences in a nonaqueous environment would be too strong to allow the nascent chain TM sequence to leave the translocon in a timely fashion. One would therefore expect different nascent chain TM sequences to vary in exactly how they bind to and are aligned with a translocon component. Our results are consistent with that expectation, because the seven TM sequences examined here yielded very different photocrosslinking patterns, even though all of the probes were located in or near the middle of the TM sequence in each case (compare Figs. 6, 10, 11, 12, 13, 14, 15). Furthermore, the extent of photocrosslinking to TRAM varied greatly, from the relatively efficient covalent reaction observed between 111p and TRAM to the absence of any photoadduct formation between Lep1p and TRAM. Although interactions between the TM flanking regions and the translocon and/or ribosome may influence TM segment positioning within the translocon, the TM sequences themselves appear to be most responsible for their placement within the translocon. This is demonstrated most clearly by the data in Fig. 13, where a single-residue change in the middle of the TM sequence elicited a dramatic

change in translocon-nascent chain photocrosslinking and hence positioning. The effect of the flanking regions will be explored further in chapter IV.

We do not yet have sufficient information and examples to discern exactly what structural features of the TM segment are being recognized and bound within the translocon. Yet the wide variety of proteinaceous environments detected by the TM sequence probes in our experiments already raise some interesting and important questions. Does every TM sequence interact with the same site in the translocon (e.g., a particular translocon α-helix)? Or do different translocon α-helices associate, either singly or in groups, with nascent chain TM sequences? Does the variable proximity of the nascent chain to TRAM indicate that nascent chains move to different locations within the translocon depending upon their TM sequences? Are the helix axes of the nascent chain TM segment and the translocon TM segment(s) parallel, so that the helices are aligned side-by-side when they interact? Or do the helices associate in the crossover manner observed with glycophorin dimers and other TM α-helices (Popot and Engelman, 2000)? The relevancy of the above questions is also shown by the recent report of High and colleagues, who found by chemical crosslinking that the first and second TM sequences of opsin have different proteinaceous environments within the translocon (Meacock et al., 2002).

It is difficult to imagine how the translocon would bind to every single TM sequence that passes into the ER membrane through the translocon, simply

because the number of different TM sequences in membrane proteins is staggering. Yet a binding process that is simultaneously promiscuous and very specific is not unprecedented. For example, the signal recognition particle (SRP) accurately selects and binds to a multitude of nascent chain signal sequences that vary greatly in sequence, hydrophobicity, and length. But whereas the SRP-signal sequence interaction involves multiple ligands and a single SRP binding site, the binding of various TM sequences to the translocon may involve several distinct sites within the translocon, as suggested above.

Phospholipids play an important role during integration. Together with other lipids, they establish a bilayer and provide a nonpolar environment that is the ultimate destination of most TM sequences. As soon as TM sequences enter the translocon, they are exposed to phospholipids (Martoglio et al., 1995; Mothes et al., 1997; Heinrich et al., 2000; data not shown). It has been postulated that this exposure results from a transient or stable exposure of the nonpolar core of the bilayer to the nascent chain in the aqueous pore of the translocon (Heinrich et al., 2000; Martoglio et al., 1995; Mothes et al., 1997). In this scenario, the translocon proteins are thought to perform primarily a gating function that allows TM sequences to interact directly with the bilayer phospholipids while in the aqueous pore and then to move into the bilayer; interactions between the nascent chain and translocon proteins are thought to be nonexistent or minimal during insertion. One recent model proposes that TM sequence insertion at the translocon is a “partitioning” effect in which TM

sequence movement into the membrane is dictated by the hydrophobicity of the TM sequence (Heinrich et al., 2000). In this model, the most hydrophobic TM sequences enter the bilayer immediately from the aqueous pore, while less hydrophobic TM sequences "dynamically equilibrate" at the aqueous-lipid interface and are inserted into the bilayer more slowly. Yet while TM sequence insertion into the bilayer is clearly driven thermodynamically by the entropy gained from moving the hydrophobic TM sequence from an aqueous to a nonaqueous environment, it is not necessary that the mechanism of insertion involves solely the TM sequence and phospholipids.

Since the more hydrophobic TM sequences move into the bilayer more rapidly than less hydrophobic sequences in the partitioning model, this model would predict that the less hydrophobic TM sequences would be retained longer at the translocon and the putative aqueous-lipid interface where they could photocrosslink to nearby translocon proteins. Yet we found that each of the seven TM sequences examined here remained adjacent to the translocon even after the nascent chain was long enough to allow the TM sequence to move away. The extents of photocrosslinking of the VSV G, O3 and O5 TM sequences to Sec61α were essentially undiminished until translation terminated (Fig. 6 and data not shown). The extent of Lep1 TM segment photocrosslinking to Sec61α decreased slowly as the nascent chain length increased (Fig. 14B), while photocrosslinking of both the TfR and O2 TM sequences to Sec61α disappeared rapidly when the probe was 90 or 93, respectively, residues from

the C-terminal end of the nascent chain (data not shown). Thus, because the photocrosslinking of these TM sequences to Sec61α did not disappear shortly after reaching the translocon, none of these TM sequences behaved as predicted by the partitioning hypothesis of Heinrich et al. (2000).

Furthermore, release of these TM sequences from the translocon and the consequent disappearance of photocrosslinking to Sec61α did not correlate with their hydrophobicities. Based on the White-Wimley values ($\Delta G_{woct} - \Delta G_{wif}$) for quantifying amino acid residue movement from the aqueous to the nonpolar phase, the free energies of transfer of these TM sequences range from –6.06 and –5.25 kcal/mole for the VSV G and Lep1 TM sequences, respectively, to the –2.17 kcal/mole of the TfR TM sequence (a more negative number indicates a more hydrophobic sequence; (White and Wimley, 1999). Even though the VSV G TM sequence is the most hydrophobic of these three, nearly all of the VSV G TM segments remained bound to the translocon in an asymmetric fashion until translation was terminated (Fig. 6). In contrast, the TfR sequence was the first to leave the translocon. Each of these results is the opposite of what is predicted by the partitioning hypothesis. We therefore conclude that the release of a TM sequence from the translocon is not dictated by its hydrophobicity, as suggested by the partitioning hypothesis, but rather is regulated by protein-protein interactions between the TM sequence and the translocon.

Although the high efficiency of TM sequence photocrosslinking to phospholipid has been interpreted as indicating that the TM sequence moves immediately into the lipid bilayer and away from the translocon, we suggest instead that the efficient photocrosslinking results from phospholipid molecules being essential structural and functional components of a translocon. Specifically, we envision that phospholipid molecules are interspersed within the translocon structure at various locations. This arrangement would provide steric advantages by allowing the mobile and flexible phospholipid acyl chains to “fill in” or occupy any space where translocon helices do not fit tightly together. Furthermore, the mobility and flexibility of the small phospholipid molecules would allow the translocon to accommodate TM segments with a myriad of sequences and surface features by simply shifting positions to occupy any free space. This flexibility would prove especially important when the structure of the translocon changes during its functional cycle (Haigh and Johnson, 2002; Hamman et al., 1997; Hamman et al., 1998; Johnson and Haigh, 2000; Johnson and van Waes, 1999) and during the lateral movement of the nascent chain TM segment through the translocon during integration. Phospholipid molecules may also be positioned along the interface between individual protein components of the translocon and perhaps along the path the TM segment travels as it moves laterally through the translocon from the aqueous pore to the lipid bilayer. Phospholipids located along such a path may not only sterically accommodate the wide variety of TM sequences, as

suggested above, but may also provide a nonpolar surface with a gradient of increasing hydrophobicity to promote TM sequence movement through the translocon towards the bilayer core after the TM segment is released by the translocon (in effect, grease the pathway). In such a structural arrangement, one can easily imagine that a TM sequence bound to a translocon protein may still be largely surrounded by and/or in contact with phospholipid molecules, and hence that phospholipids would be the primary photocrosslinking target even when the TM segment is bound and adjacent to a translocon protein.

Does the presence of the photoprobe interfere with TM segment processing at the translocon? Since the membrane proteins examined here are successfully integrated into the bilayer in the proper orientation with or without the probe, the probe has no apparent effect on the integration of these proteins. The εANB-Lys residue incorporated into the TM segment appears benign, both because it is very nonpolar (the modification eliminates the charged amino group and leaves behind an amide-linked aromatic probe moiety and an aliphatic lysine side-chain) and because the long flexible lysine side-chain would allow it, if necessary, to bend and minimize or eliminate any steric conflict. Although the presence of an εANB-Lys in a TM segment may certainly alter the exact manner in which a particular TM segment interacts with the translocon, the observed differences in the photocrosslinking from different probe locations show that the presence of a probe in the TM segment does not force the TM segment to orient in a particular direction within the translocon.

Why are protein-protein interactions involved in TM segment insertion? Interactions between the nascent chain and the translocon are presumably required to ensure that each membrane protein is properly oriented and assembled. For example, accurate integration of multi-spanning membrane proteins may require the translocon to bind to a TM segment to determine its orientation in the bilayer and thereby ensure that the succeeding TM sequence is inserted in the opposite orientation. Protein-protein interactions may also be required to effect the integration of an amphipathic TM α-helix of a channel or pore protein because a hydrophilic or charged TM sequence is intrinsically less thermodynamically stable in the bilayer than is a typical hydrophobic TM sequence. Hence, an amphipathic TM segment may have to be held within the translocon by protein-protein interactions until association with other TM segments of the membrane protein can stabilize its presence in the nonpolar core of the bilayer. In addition to other possibilities, protein-protein interactions may also be necessary to retain TM segments within or adjacent to the translocon so as to facilitate the assembly and/or folding of a multi-spanning membrane protein. The retention of a TM sequence may even play a role in the cell's quality control processes by providing a mechanism for holding the nascent or complete chain of a misfolded or misassembled membrane protein at the translocon prior to retrotranslocation into the cytosol for degradation (Johnson and Haigh, 2000). Whatever the case, the translocon does not appear to be simply a hole in the membrane that periodically opens laterally to expose

TM sequences to the bilayer. Instead, the translocon apparently participates actively in effecting and regulating nascent chain movement into the ER membrane.

CHAPTER IV

INVESTIGATING THE NATURE OF THE INTERACTION BETWEEN THE NASCENT CHAIN TM SEGMENT AND THE TRANSLOCON

Experimental Design

Now that it is established that a TM segment is held by the translocon it is important to investigate the nature of this interaction. Do the flanking regions have any detectable affect on the interaction between a TM segment and the translocon proteins? Where in the TM segment do these interactions occur? The general perception of membrane protein integration is that a TM segment is perpendicular to the plane of the bilayer and that the interaction of any given TM segment with the machinery of the translocon would run the length of that particular TM segment. Of the membrane proteins whose structure is known, many contain at least one TM region that is tilted with respect to the plane of the bilayer. This raises the question, how are TM segments interacting with the translocon proteins? Do TM segments exit the ribosomal tunnel and directly abut the TM segments of the translocon in a parallel fashion? Or is there an angle between the two TM segments as has been shown by (MacKenzie and Engelman, 1998; MacKenzie et al., 1997)?

By placing photoprobes in the center of two different TM segments we have tried to explore the above questions. We have designed one of these TM segments with three different flanking regions and asked what effect changing

the flanking regions had on TM-translocon interaction. The other TM segment was used to scan the TM segment to see where the interaction with the translocon occurs by placing probes at eight different positions in the center of the TM sequence and observing which sites yielded photoadducts.

Is the Interaction between the Translocon and a TM Segment Altered by Changing the Flanking Sequences?

To investigate the role, if any, the flanking regions play in integration we designed three different constructs that contained the H1 TM segment of leader peptidase surrounded by three different flanking regions. The first series of constructs derives from the 111p protein, where Lep1 was placed in the position of the VSVG TM segment in the 111p protein to yield a protein designated 111/Lep1. Thus, the new polypeptide contained the preprolactin signal sequence followed by the signal-anchor portion of leader peptidase. The second set of constructs has a 17 amino acid addition to the N-terminal side and a deletion to the C-terminal side of Lep1 compared to the wild-type protein to yield a protein designated Lep-SA. The third set of constructs contains the native leader peptidase flanking regions and was designated Wild-Type Lep. If the flanking regions influence the interaction of the TM segment with the translocon proteins, then one would expect to detect a difference in the pattern of photocrosslinking observed in the three different constructs. If, however, the flanking regions have no affect on the interaction between the TM segment and the translocon, then the results from all three sets of constructs should be the

same. Probes were placed in the same position of the TM segment as were tested in the Lep1 protein in chapter III. Photocrosslinking to Sec61α was then examined.

As was observed for the conctructs in chapter III, all three proteins showed an asymmetric pattern with crosslinking to Sec61α, meaning all three TM segments are held within the translocon. In addition, much to our surprise, the same pattern of crosslinking was observed for all three constructs (Fig. 17). That is, when the probe was placed at position 10 and 11 of the TM segment in each set of constructs photocrosslinking to Sec61α was observed. However, when the probe was placed at position 9, no photocrosslinking was observed. Thus, in every case the, not only is TM segment held in the translocon, but in each construct it is being held in what appears to be the same orientation with respect to Sec61α, despite the different flanking regions and the differences in location within the nascent chain. Therefore, the position which a TM segment is held within the translocon is specific to the primary structure of the TM segment.

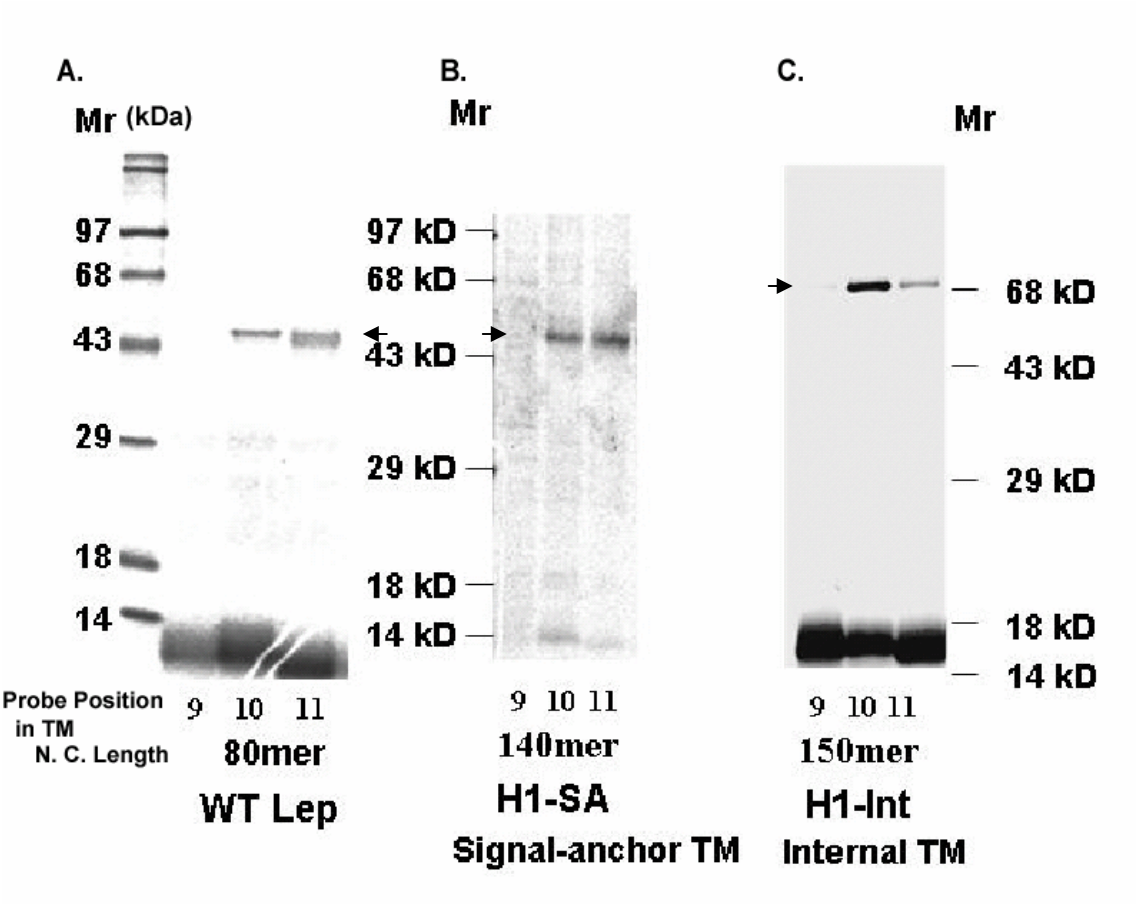

Figure 17 Crosslinking to Sec61α with the H1 TM Segment Using Different Flanking Regions.

Integration intermediates were prepared, photolyzed, and immunoprecipitated with antibodies to Sec61α. Samples contained (A) 80-residue nascent chains of Lep(9,10,11), (B) 140-residue nascent chains of LEP1 (9,10,11) or (C) 150-residue nascent chains of 111/Lep1 (9,10,11). Photoadducts containing Sec61α are identified by the arrowhead.

If the flanking regions have no effect on the binding of a TM segment to the translocon, do they influence the point at which a TM segment leaves the translocon. Experiments were performed using the 111/Lep1 and Lep1 constructs at longer lengths and photoadducts were immunoprecipitated with antibodies against Sec61α. It was found that in both cases the TM segment stayed in contact with Sec61α until synthesis of the polypeptide was complete (Fig. 18). Thus, not only is the influence of the flanking regions on the TM-translocon interaction minimal, but there appears to be no affect of the flanking regions on the release of a TM segment into the lipid bilayer.

To explore whether or not a TM segment interacts with a translocon protein in a parallel fashion or not, a single probe was placed at eight different positions within the 111p TM sequence, from 111p(71) to 111p(78). If the nascent chain and translocon TM segments were parallel to each other, then one would expect that all probe locations on the side of the nascent chain TM segment α-helix that faces the translocon TM segment would crosslink the two polypeptides no matter the depth of the position. This would mean that cross-links should be seen periodically (every 3-4 residues) along the entire length of the nascent TM segment. Alternatively, if the two TM segments were interacting at an angle, then crosslinks would only be found in one portion of the nascent chain TM segment. The further away the probe was from the point of interaction, then the lower the probability that probe would react with that particular TM segment of the translocon.

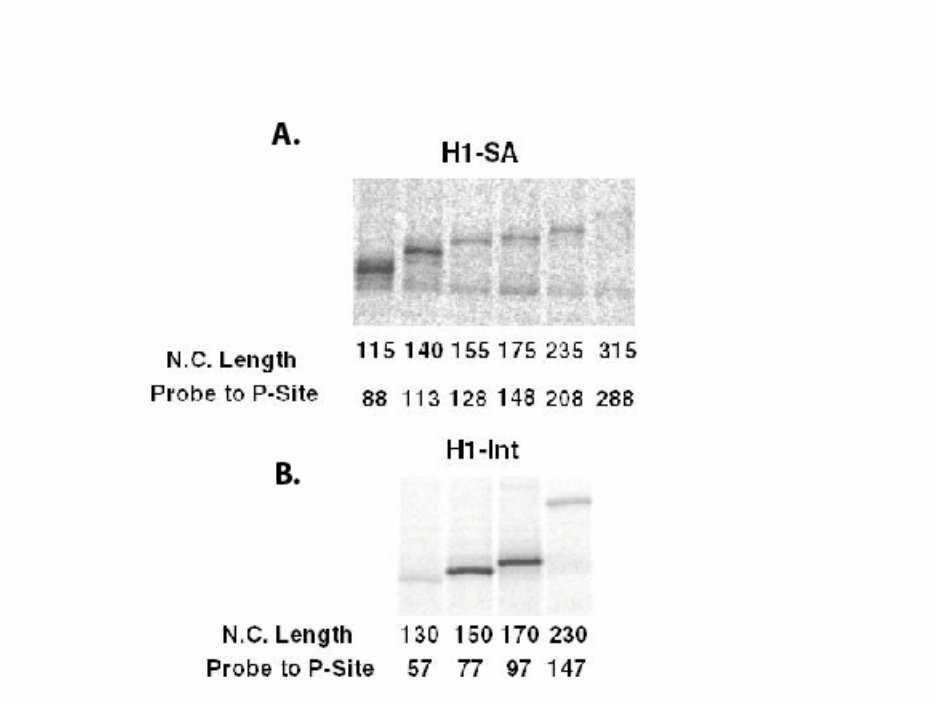

Figure 18 Crosslinking of the H1 TM Segment to Sec61α at Different Lengths of Nascent Chain.

Integration intermediates were prepared as described in Chapter II using [^{35}S]Met, εANB-Lys-tRNA^{amb}, and truncated mRNAs that yielded nascent chains of (A) LEP1(10) at 88, 113, 128, 148, 208, and 288 amino acids in length and (B) 111/Lep1(10) at 57, 77, 97, 147 amino acids in length.

Using a length of 130 amino acids, photocrosslinking and immunoprecipitation with antibodies to Sec61α yielded the data shown in Fig 19. It can be seen that photoadducts were only formed when the probes were placed at positions 75, 76, or 77 within the nascent TM segment. This result indicates that the interaction between the nascent chain TM segment and the translocon proteins does not span the entire lipid bilayer, but is isolated to the center of the TM segment. This result further implies that the nascent chain and Sec61α TM segments must not be parallel to each other at this stage of integration.

Discussion

The data presented in this chapter highlight some of the characteristics of the interaction between an integrating nascent TM segment and Sec61α. By using a higher resolution approach, we have shown that a nascent chain TM segment does not align in a parallel fashion with the Sec61α TM segment to which it binds. This would be consistent with the association of TM helices characterized by Engelman and colleagues (Popot and Engelman, 2000), in which two TM segments bind by partially folding around each other to create an interface that is at an angle. Since we have shown in the previous chapter that the nascent TM segment is bound to a translocon TM segment(s), this result would be consistent with the binding of the nascent chain TM segment to a Sec61α TM segment. In addition, for TM sequences whose final positioning in the membrane is tilted with respect to the plane of the bilayer, this result may

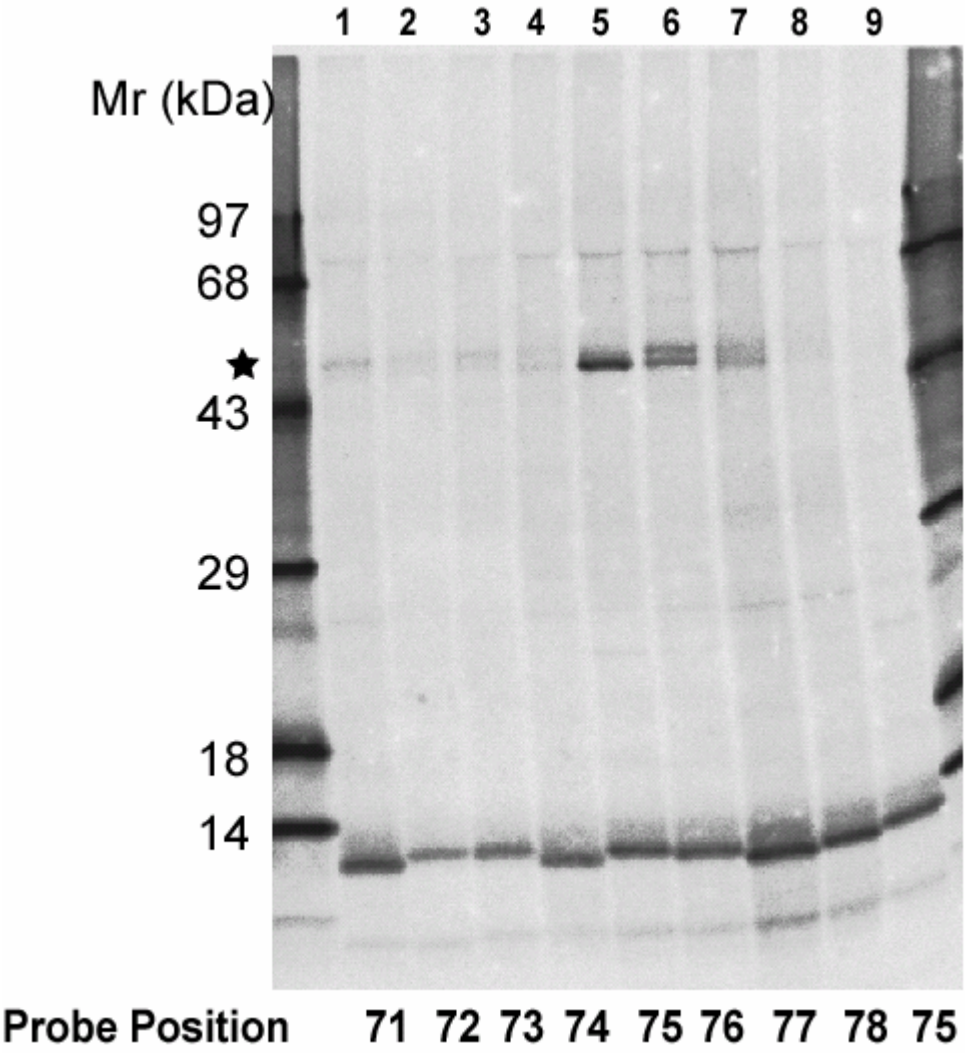

Figure 19 Crosslinking of 111p to Sec61α from Different Probe Positions in the TM Segment.

Integration intermediates containing 130-residue nascent chains of 111p(71,72,73,74,75,76,77,78) were prepared, photolyzed and immunoprecipitations with antibodies to Sec61α performed. Lane 9 received unmodified Lys-tRNA instead of εANB-Lys-tRNA. Photoadducts containing Sec61α are indicated by the star.

mean that the angle they have at maturity may be achieved early on in the integration process. At minimum, this result shows that the interaction between TM segments with translocon proteins does not restrict integrating TM segments to being parallel to each other within the bilayer, although further study will be required to investigate all the possible angles of interaction between nascent chains and the TM segments of the translocon. The second finding demonstrated in this chapter is that flanking regions appear to have little bearing on how a particular TM segment interacts with Sec61α. Nascent chain TM segments therefore appear to be handled individually by the translocon, without detectable influence from the flanking sequences. This result further indicates that nascent chain TM interaction does not derive from the flanking regions, an idea reinforced by the proline mutation in opsin 5 presented in chapter III. Flanking regions may serve other purposes in the final structure and function of a membrane protein, but their influence on the placement of a nascent TM segment in the translocon during integration appears to be minimal.

CHAPTER V

THE TIMING OF TM SEQUENCE INTEGRATION INTO THE ER MEMBRANE IN POLYTOPIC MEMBRANE PROTEINS

Experimental Design

All at Once, Sequential, or Pair-Wise

Since an individual TM segment binds directly to translocon proteins and its movement is controlled by protein-protein interactions (chapter III), it is important to determine how TM segments interact with translocon proteins when polytopic membrane protein integration causes a series of TM sequences to enter the translocon. How is a TM segment's movement influenced by the presence of other hydrophobic regions in the same polypeptide? Do two TM segments in the same polypeptide interact simultaneously with the translocon? Does the binding of one TM sequence to the translocon affect the binding of the other to translocon proteins? These are only a few of the important mechanistic issues that have not been resolved experimentally.

There are currently three models that have been proposed for how TM sequences integrate into the ER membrane. The first model, the 'all at once model' (Borel and Simon, 1996), proposes that all TM segments are held within the translocon until synthesis of the nascent chain is complete. This model predicts that the first TM sequence in the nascent protein (TM1) should be observed adjacent to translocon proteins even after the subsequent TM sequence (TM2) and later ones reach the translocon, and this condition should continue until the nascent chain is released from the ribosome. The second model, called the 'pair wise-model', predicts that two TM sequences will interact

within the translocon and then move into the lipid bilayer as a pair (Skach and Lingappa, 1993; (Lin and Addison, 1995); Heinrich et al, 2003). In this instance, TM1 will be found in the translocon, until TM2 enters the translocon, at which point TM2 and TM1 will move into the lipid bilayer together. The third model is called the sequential model (Do et al., 1996; Meacock et al., 2002). The sequential model proposes that TM sequences are integrated into the lipid bilayer singly and in sequence. In this model, TM1 is found inside the translocon until TM2 emerges from the ribosome and enters the translocon at which point TM1 leaves the translocon by itself. Since TM2 is thought to displace TM1 from the translocon, the entrance of TM2 into the translocon will correspond with the movement of TM1 away from the translocon. These three models are depicted and summarized in Figure 20.

To discern between these models, the relative timing of TM entry into and exit from the translocon must be determined. To achieve this experimentally, one must monitor what is adjacent to any given TM sequence as the length of the nascent chain increases. The photocrosslinking approach introduced in Chapter III is a perfect technique for this purpose. A photocrosslinker can be introduced into each TM region of a multi-spanning membrane protein as before. One can then determine what each TM segment crosslinks with as it integrates into the lipid bilayer. The relative timing of the appearance and disappearance of photocrosslinks to translocon proteins will then indicate where each TM is located at each stage of integration.

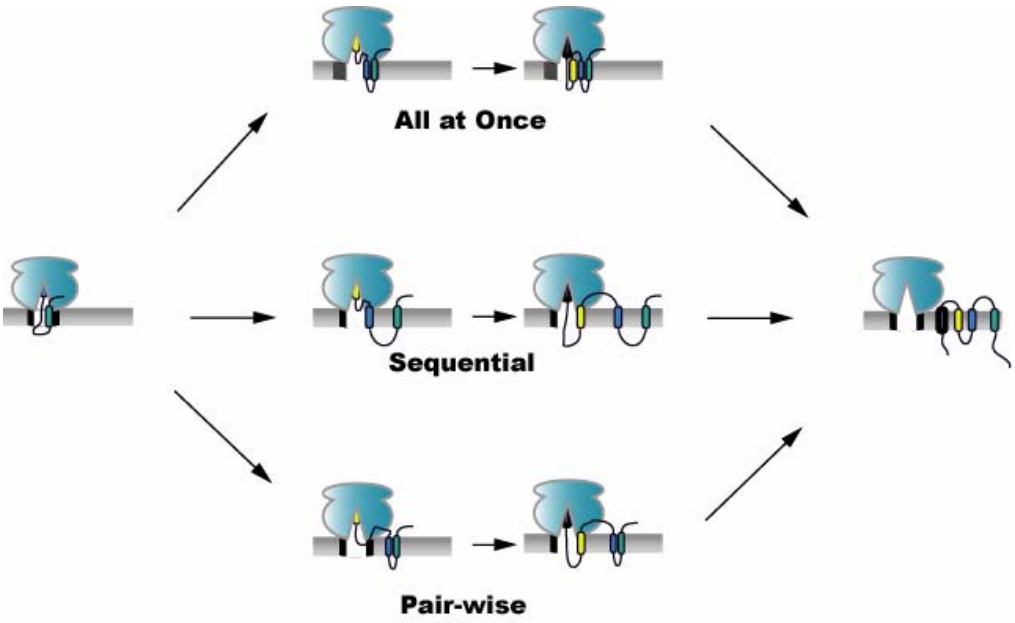

Figure 20 Models for Multi-Spanning Integration.

The first TM segments of all nascent membrane proteins are initially bound to the translocon. The all at once model proposes that all TM segments are bound by the translocon upon synthesis and held until the complete polypeptide is made and released. The sequential model proposes that the entry of each succeeding TM segment into the translocon will cause the preceding TM segment to be released from the translocon. Thus, only one TM will be bound by the translocon at any given stage of integration. The pair-wise model states that TM sequences in multi-spanning membrane proteins will move into the lipid bilayer in pairs.

Integration Intermediates

Integration intermediates were prepared in vitro as described in Chapter II. The membrane proteins used in this study are shown in Fig. 21. The proteins that contain a cleavable signal sequence use the signal of the preprolactin (pPL) protein. In addition, we also examined a membrane protein that contained a signal-anchor sequence that serves both as a signal sequence to target the nascent chains to the translocon and as a TM sequence to integrate and anchor the protein in the ER membrane. Integration intermediates were synthesized with photoreactive probes as before, then photolyzed to determine what translocon proteins, if any, were adjacent to the labeled nascent chain TM sequence.

Photocrosslinking of a Single TM Segment

To examine the timing of TM segment passage through the translocon, it was important to first establish the behavior of a single TM domain and its proximity to translocon proteins during integration. When crosslinking experiments were performed using 111p(75)$_{130,150,170,230}$ and photoadducts were detected by immunoprecipitation using antibodies to Sec61α it was found that at all lengths tested 111p(75) continued to crosslink to Sec61α. Crosslinking continued until 230 amino acids (Fig. 22). The full lenth polypeptide is 232 residues, thus the TM segment of 111p(75) does not leave the proximity of Sec61α until after translation is complete. This result confirms a result seen in Chapter III, but here demonstrates it in a single experiment. Therefore, a single

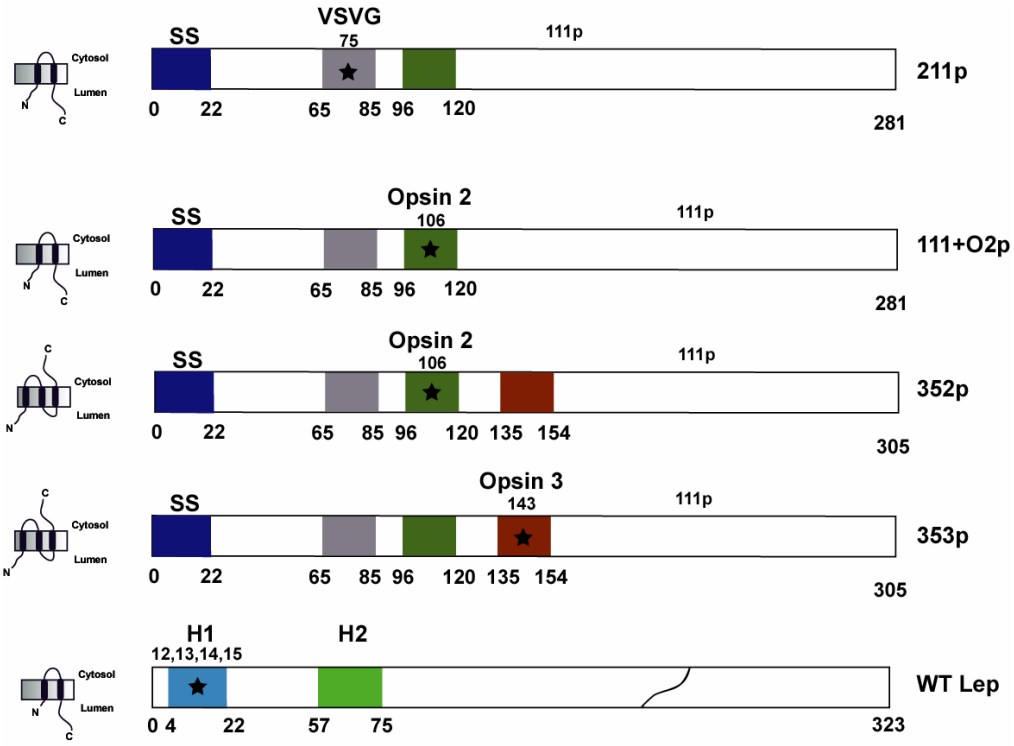

Figure 21 Multi-Spanning Membrane Proteins.

As in Chapter III, most of the membrane proteins examined here have a preprolactin-derived signal sequence (SS) that is cleaved by signal peptidase. A single Lys or amber stop codon in each coding sequence directs the probe (star) to the indicated position in a TM segment. VSV G, is the TM sequence of the VSV G protein. H1 and H2 are the first and second TM sequences in leader peptidase, while the second and third TM sequences in opsin are designated Opsin 2 and Opsin 3.

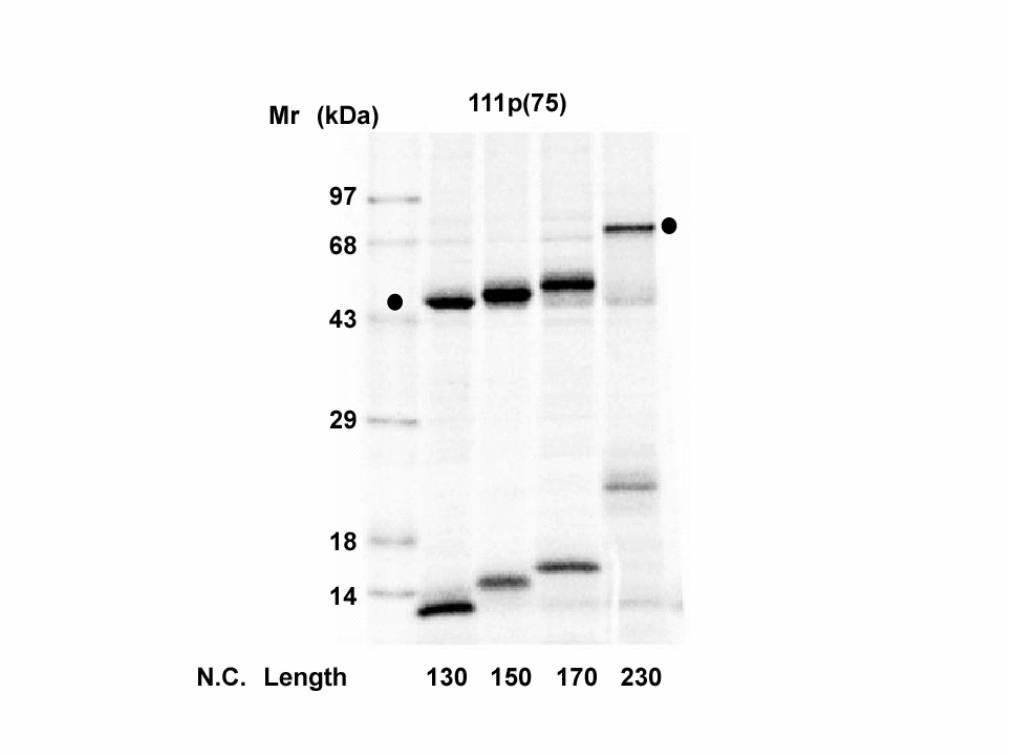

Figure 22 Crosslinking of 111p(75) to Sec61α.

Integration intermediates were prepared in parallel using [^{35}S]Met, εANB-Lys-tRNA^{Lys}, and truncated mRNAs that yielded nascent chains of 111p(75) with lengths of 130, 150, 170, and 230 amino acids. After photolysis, photoadducts containing Sec61α were purified by immunoprecipitation and SDS-PAGE (indicated by black circles). Molecular mass standards were electrophoresed to obtain the apparent molecular mass (Mr) values in kilodaltons (kDa).

TM sequence will stay associated with the translocon via protein-protein interactions until synthesis is terminated.

A Second TM Sequence Displaces the Preceding TM Sequence from the Translocon

To investigate what affect a second TM segment has upon TM1 crosslinking, the second TM sequence of bovine opsin (O2) was placed behind the VSVG TM sequence in 111p(75) to create a construct designated 211p(75) see fig 21. This construct is identical to 111p(75) except that it contains a second hydrophobic TM sequence behind the first TM segment. Integration intermediates of 128, 138, 148, and 158 amino acids were analyzed for reactivity to Sec61α (fig. 23). At 128 amino acids, TM1 is outside the ribosomal tunnel, but TM2 has not yet emerged. As with the 111p(75) construct containing only one TM domain, photoadducts to Sec61α were observed. However, at longer lengths of 211p(75), as TM2 emerges from the ribosomal tunnel, crosslinking of TM1 to Sec61α almost disappeared. A similar result was observed with TRAM (data not shown). These results show that, the second TM domain causes TM1 to move away from the translocon before the nascent chain is completely translated.

A higher resolution experiment (in terms of nascent chain length), shows the rapid loss of Sec61α crosslinking over a span of ten amino acids (fig 24). Such a rapid disappearance suggests that the process of integration is controlled by protein-protein interactions, because the departure of the TM

sequence from the translocon is precisely timed relative to the entry of TM2 into the translocon. The all-at-once model predicts that TM1 will continue to be adjacent to the translocon after TM2 enters the translocon. As can be seen in fig. 24 lane 5, crosslinking of TM1 to Sec61α is severely reduced at 150 amino acids. In addition, no photoadducts were observed in the totals when the nascent chain was 150 residues or longer, (data not shown). The immediate conclusion is that the all-at-once model does not accurately explain the results obtained in this experiment. Instead, the results lend credence to the sequential mechanism that predicts that TM2 will displace TM1 from the translocon.

If each TM sequence is held in the translocon until the subsequent TM sequence moves into the translocon, then lengthening of the loop between TM1 and TM2 should allow TM1 to crosslink to translocon proteins at longer lengths of nascent chain than was seen with 211p(75). To test this prediction, a construct was made that contained a 40 amino acid insert between TM1 and TM2, thereby yielding a loop size of approximately 50 amino acids. This construct was designated 211L43p(75). When this construct was translated, photolyzed, and immunoprecipitated with antibodies to Sec61α, TM1 was found (fig. 25) to react covalently with Sec61α at longer nascent chain lengths then observed with the shorter loop.

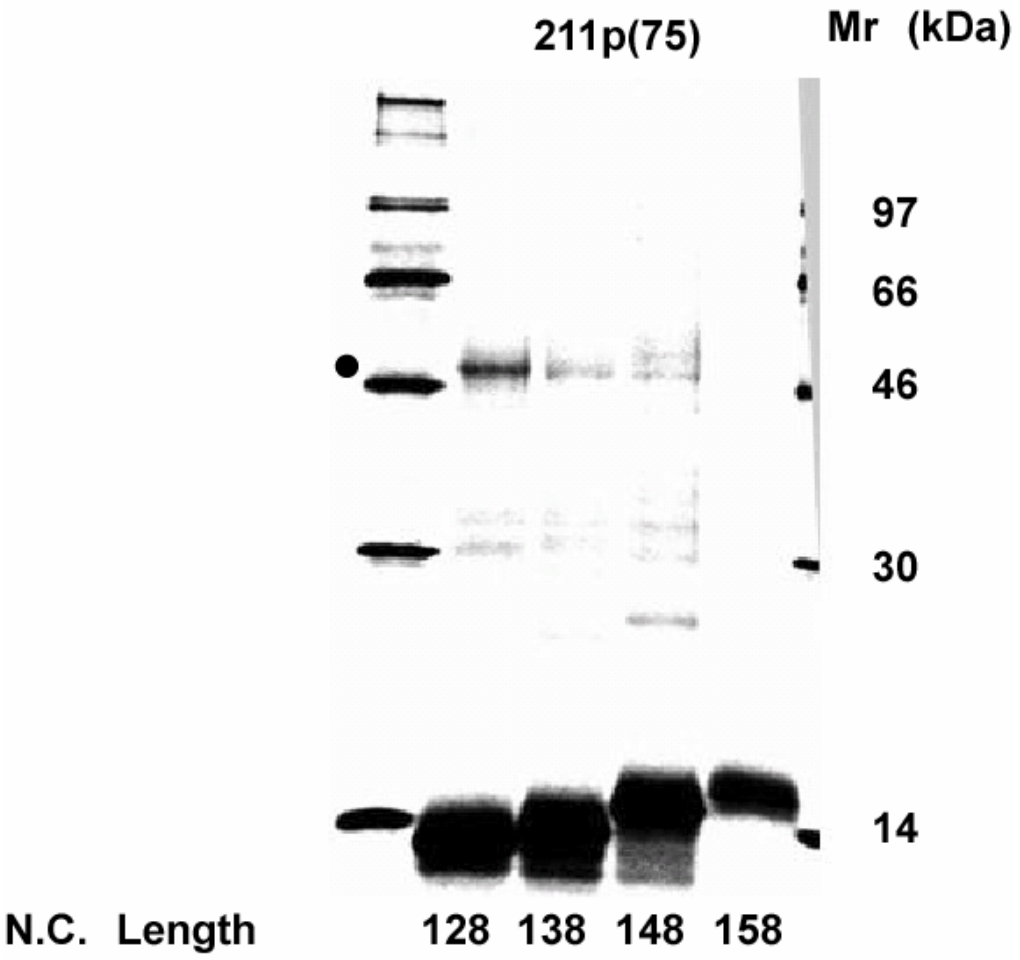

Figure 23 Photocrosslinking of 211p(75) to Sec61α.

Integration intermediates containing 128-, 138-, 148-, or 158-residue nascent chains of 211p(75) were prepared in parallel with εANB-Lys-tRNA^{Lys}, and were analyzed as described in Chapter II. Photoadducts containing Sec61α are indicated by the black circle.

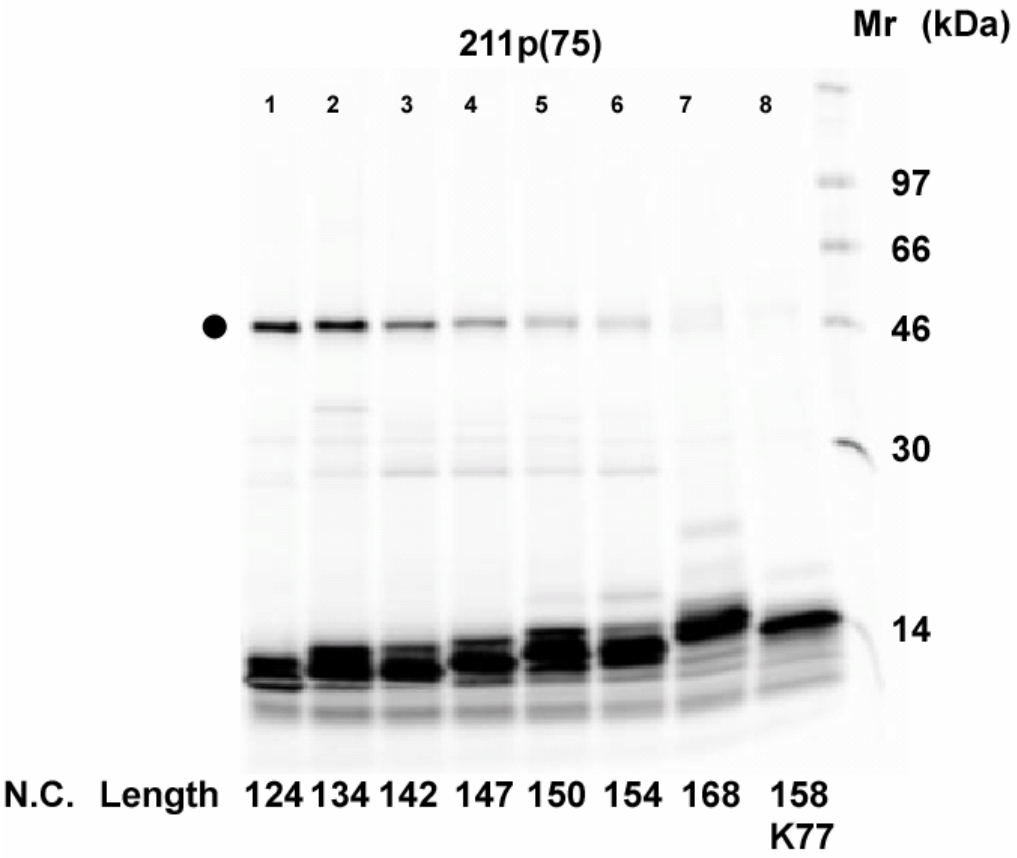

Figure 24 Photocrosslinking of 211p(75) and 211p(77) to Sec61α at Higher Resolution.

Integration intermediates were prepared in parallel using [^{35}S]Met, εANB-Lys-tRNA^{Lys}, and truncated mRNAs that yielded nascent chains of 211p(75) with lengths of 124, 134, 142, 147, 150, 154, and 168 residues. After photolysis, photoadducts containing Sec61α (black circle) were purified by immunoprecipitation and SDS-PAGE. The lane labeled K77 shows a sample containing the construct 211p(77) with a nascent chain of 158-residues was used in the same experiment as 211p(75).

In fig. 24, lane 6, the nascent chain is 154-residues in length yet TM1 can no longer crosslink with Sec61α due to the entry of TM2 into the translocon. Compare this result with fig 25, lane 3, where TM1 shows strong crosslinking to Sec61α, at a nascent chain length of 157 amino acids. In this construct, when the nascent chain is 157 residues in length TM2 has not been completely synthesized and thus not emerged from the ribosomal tunnel. Therefore, as predicted, TM1 can crosslink to translocon proteins at longer lengths of nascent chain. Thus, the displacement of TM1 by TM2 is elicited by the entry of TM2 into a translocon containing TM1, irrespective of the length of the cytoplasmic loop that separates TM1 and TM2.

The pair-wise model predicts that TM2 and TM1 will move out into the lipid bilayer together. To determine whether this indeed occurs, the construct 111p+O2 was used. This construct, introduced in Chapter III, contains the VSVG TM sequence followed by the second transmembrane segment of bovine opsin as TM2. This construct contains a lysine codon in the middle of the second TM sequence so that a photoprobe can be incorporated into TM2. Photocrosslinking experiments and immunoprecipitation with antibodies to Sec61α were performed, as usual. As shown in fig 26A, lane3**,** TM2 continues to crosslink to Sec61α even when the nascent chain is 178 amino acids in length. Comparing this result with fig. 24, lane 5, where TM1 has moved away from the translocon at 150 amino acids, it is clear that TM2 remains adjacent to

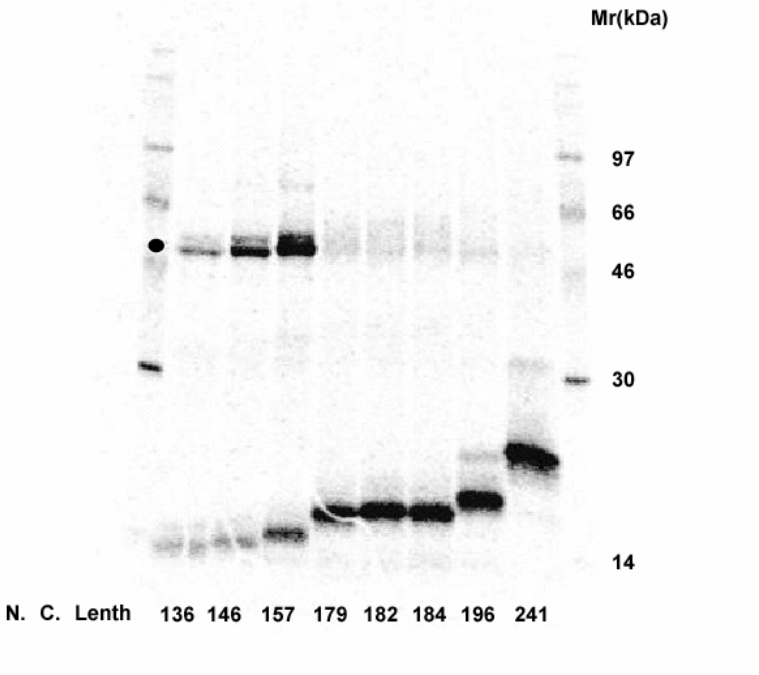

Figure 25 Crosslinking of 211L43p(75) to Sec61α.

Using the construct 211L43p(75), integration intermediates containing 136-, 146-, 157-, 179-, 182-, 184-, 196-, or 241- residue nascent chains were prepared, photolyzed and immunoprecipitations with antibodies to Sec61α performed. Photoadducts containing Sec61α are indicated by the black circle.

Sec61α after TM1 has moved away. Therefore, TM2 and TM1 are not inserting as a pair into the lipid bilayer as predicted by the pair-wise model. Instead, TM2 remains in the translocon for at least another 28 amino acids after TM1 has lost crosslinking to Sec61α (fig 24) and TRAM (data not shown).

Whereas TM1 remained in the translocon until translation was complete (fig 22), TM2 does not appear to stay in the translocon until nascent chain synthesis is complete (see the disappearance of photoadduct in lane 4, fig. 26A). This may be explained by the short tether of 11 amino acids between TM1 and TM2, and by TM1 pulling TM2 out of the translocon as TM1 moves into the bilayer. If this were to occur, increasing the length of the tether may diminish the influence of TM1 on the timing of TM2's departure from the translocon. To test whether TM1 is dragging TM2 into the bilayer, we engineered a loop that is 41 amino acids long between the two TM sequences that might allow TM1 to leave the translocon without forcing TM2 to leave the translocon at the same time as previously observed. When photocrosslinking and immunoprecipitation were performed as before, it can be seen in Fig. 26B (lanes 4-6) that TM2 leaves the translocon when the nascent chain is longer then 219 residues in length. This is the about the same point in the integration process as was observed with the construct containing the short tether, (fig. 26A, lane 3, 219aa - 41aa = 178aa). This result is surprising, with a large loop between TM1 and TM2, TM1 should have plenty of tether to diffuse into the bilayer without dragging TM2 out of the translocon. The fact that TM2 appears to leave at approximately the equivalent stage of integration implies that something other than the pulling of TM1 is driving TM2's departure from the translocon.

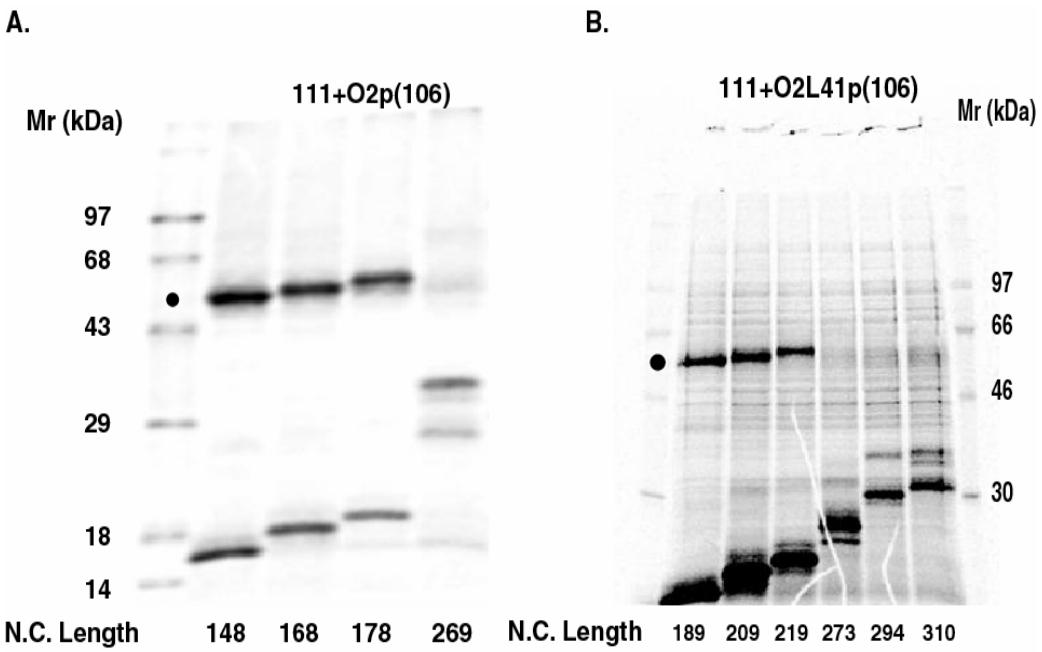

Figure 26 Photocrosslinking of 111+02p and 111+O2L41p to Sec61α.

Integration intermediates were prepared, photolyzed, and immunoprecipitated with antibodies to Sec61α as in Fig. 25. Samples contained A) 148-, 168-, 178-, or 269-residue nascent chains of 111+O2p(106) or B) 189-, 209-, 219-, 273-, 294-, or 310 residue nascent chains of 111+O2L41p(106). Bands containing photoadducts to Sec61α are labeled with black circles.

The Second and Third TM Segments Will Move out of the Translocon as a Pair

Since TM2 appears to stay in the translocon after TM1 has integrated into the lipid bilayer (figs 26 A and B) the next question is whether the entry of TM3 into the translocon will cause TM2 to leave the translocon, or whether TM2 and TM3 will leave the translocon together. The pair-wise model predicts that TM2 and TM3 will insert into the bilayer together, and thus both TM2 and TM3 photocrosslinking to translocon proteins will be lost when the nascent chain reaches a particular length. The sequential model predicts that as soon as TM3 emerges from the ribosome, TM2 crosslinking to the translocon will disappear, and instead TM3 will react with translocon proteins.

To understand how TM2 and TM3 integrate into the membrane, a construct containing the VSV G TM segment followed by Opsin 2 and Opsin 3 was designed and designated 352(106)p. This construct contains a lysine in the second TM region (Opsin 2) for incorporation of a photoprobe. Using the 352(106)p construct, with the probe placed in the second TM segment, photocrosslinking experiments were performed with nascent chains that were 138, 172, 182, 192, and 223 amino acids in length. As shown in fig 27 (lanes 2 and 3), TM2 reacted with Sec61α as soon as it emerged from the ribosome. However, at 192 amino acids, TM2 no longer crosslinked to Sec61α (Fig. 27 lane 4). At this length, TM3 is expected to have entered the translocon, which suggests that TM3 caused TM2 to be released from the translocon.

As mentioned above, if a probe placed in TM3 is able to react with Sec61α after TM2 crosslinking to the translocon is lost, then the TM sequences would be integrating in a sequential manner. If, however, a probe in TM3 did not react with translocon proteins after TM2 was observed to move out of the translocon, then the TM sequences would have moved out as a pair into the lipid bilayer and the pair-wise model would more accurately describe the mechanism of TM2 and TM3 integration. To distinguish between these two models, the same construct [352p] was used, except that the probe was placed within TM3 instead of TM2 to yield 353p(143). When crosslinking and immunoprecipitation experiments were carried out, it can be seen in fig 28A (lanes 3 and 4) that TM3 does indeed react with Sec61α. Yet TM3 crosslinking to Sec61α does not persist. There are no significant photoadducts after TM2 has moved away from the translocon (lanes 5-8), and the lengths at which TM2 and TM3 crosslinks to Sec61α are lost are essentially identical (compare figs. 28A and B lanes 5 and 6 respectively). This result implies that TM2 and TM3 are moving out of the translocon as a pair.

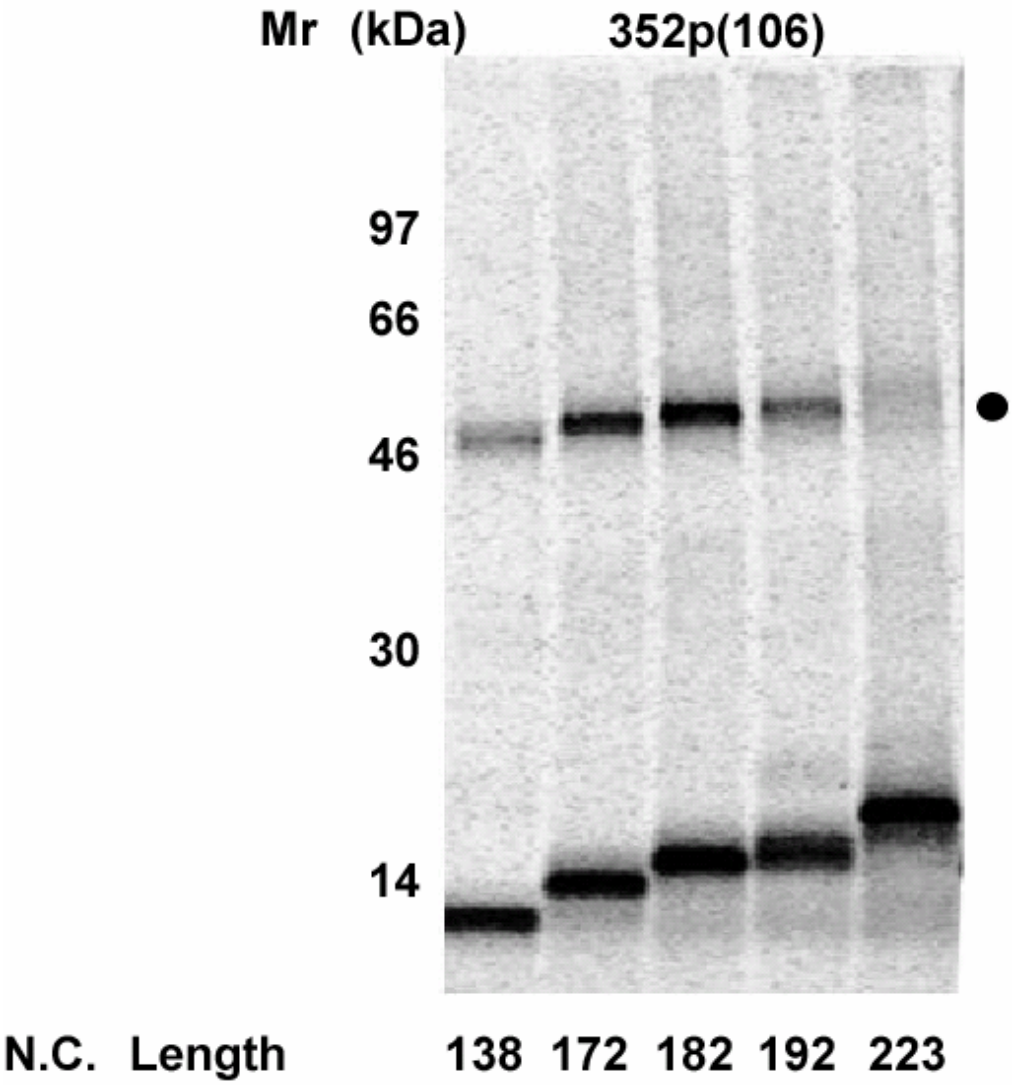

Figure 27 352(106)p Crosslinking to Sec61α.

Integration intermediates were prepared, photolyzed, and immunoprecipitated with antibodies to Sec61α as in Fig. 22. Samples contained nascent chains of 352(106)p that were 138, 172, 182, 192, and 223 residues in length. Photoadducts to Sec61α are indicated by the black circle.

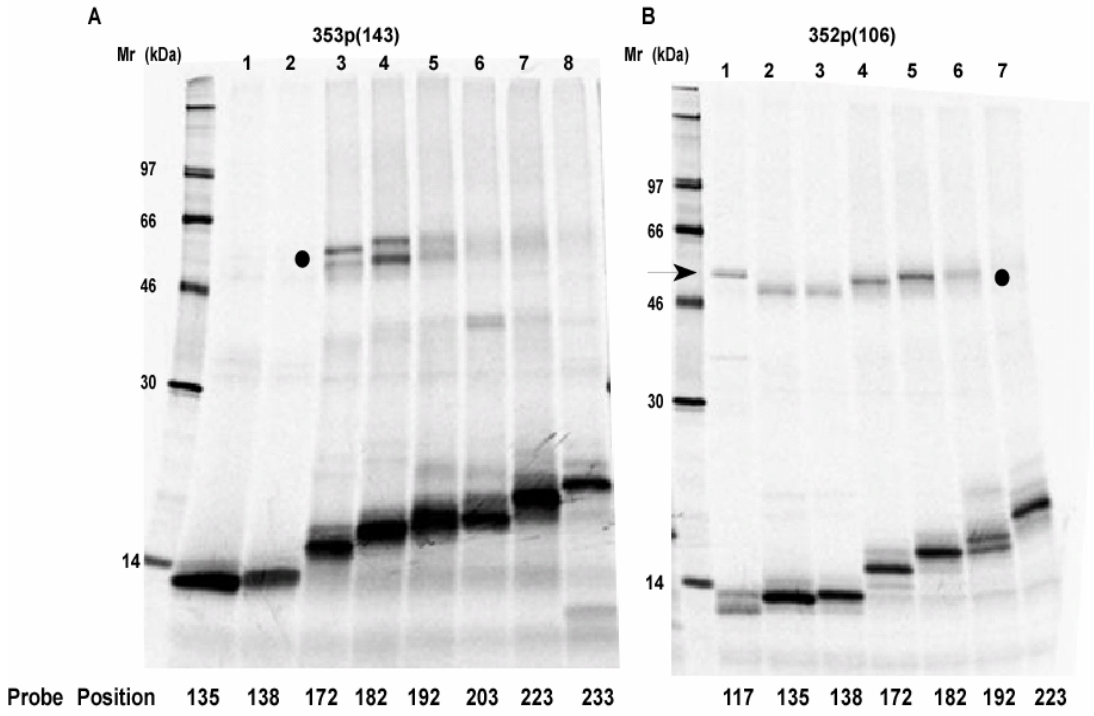

Figure 28 Crosslinking of 353p(143) and 352p(106) to Sec61α.

Integration intermediates containing (A) 353p(143) with lengths of 135, 138, 172, 182, 192, 203, 223, and 233 residues or (B) 352p(106), or 111p(76) nascent chains with lengths of 117, 135, 138, 172, 182, 192, and 223 residues residues were examined as in Fig. 22. After photolysis, photoadducts containing Sec61α were purified by immunoprecipitation and SDS-PAGE and are identified by the black circles. The photoadduct labeled with an arrowhead is a ribosomal crosslink that is present in the absence of membranes and that is weakly recognized by the affinity-purified Sec61α antibodies.

Signal-Anchor Segments Are also Displaced from the Translocon by TM2

The above constructs all contain a cleavable signal sequence that targets the ribosome•nascent chain complex to the translocon. Signal-anchor segments are unique TM sequences that serve as the signal sequence for signal-anchor proteins, are synthesized and exposed to the cytosol where they are recognized and bound by SRP. The TM segments examined so far in this chapter have all been internal TM segments that enter the translocon directly from the ribosomal tunnel. The contrast between cytosolic exposed signal-anchors and internal TM sequences raises the possibility that a signal-anchor sequence may use a different pathway for integration that would not have the signal-anchor sequence displaced by a TM2. To test whether signal-anchor sequences also follow a similar pattern of insertion as internal TM segments, probes were placed within the signal-anchor (H1) sequence of the leader peptidase protein. Photocrosslinking and immunoprecipitation experiments were performed using antibodies to Sec61α. As shown in fig 29, (lanes 2, 3, 6, and 7), H1 was adjacent to Sec61α when the nascent chain was 80 or 92 amino acids in length. Crosslinking efficiency was ~16% at these lengths of nascent chain. From previous experiments in Chapter III, it is known that, in the absence of H2, H1 crosslinking can be detected until the completion of synthesis of the nascent chain. However, with the addition of H2, crosslinking to Sec61α is lost by the time the nascent chain reaches 117 amino acids in

length (fig. 29, lanes 9-12). Thus, signal-anchor containing proteins also use a sequential mechanism for the integration of hydrophobic TM sequences into the bilayer after the signal-anchor sequence is inserted. At this point, it is unclear why our results differ from those of Heinrich and Rapoport (2003).

Displaced TM Sequences Leave the Translocon

An alternative interpretation of the above results is that a displaced TM segment does not leave the translocon, but moves to another location within the translocon in which the photoprobe extends away from the translocon and can no longer react with translocon proteins. For example, if the TM segment were to rotate and move such that the lysine side chain containing the probe now faced away from the translocon protein that is binding the TM segment, then no photoadduct would be formed, even though the TM segment is still bound to the translocon. If this were the case, then one would predict that moving the probe to different faces of the TM α-helix should result in one or more of the probe positions reacting with the translocon protein if the TM segment is still bound to the translocon. Hence, one can, in principle, detect whether or not the TM sequence has left the translocon by testing all sides of the TM α-helix for crosslinking to Sec61α.

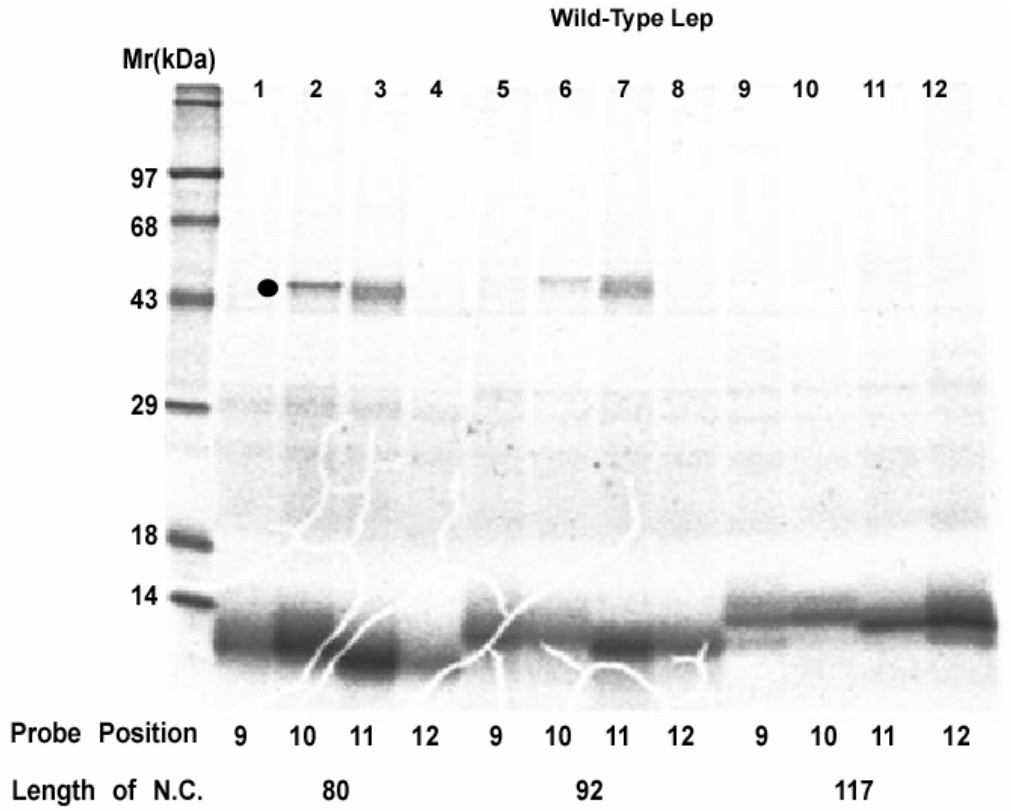

Figure 29 Photocrosslinking of Wild-type Lep to Sec61α.

Integration intermediates were prepared using [^{35}S]Met, εANB-Lys-tRNA^{amb}, and truncated mRNAs that yielded nascent chains of: WT Lep(9), WT Lep(10), WT Lep(11), and WT Lep(12) at 80, 92, and 117 residues. After photolysis, photoadducts containing Sec61α were purified by immunoprecipitation and SDS-PAGE (indicated by black circle).

To test whether the displaced TM sequences were indeed leaving the translocon or just in a position where they could no longer react, we positioned the probe in the TM helix at different locations and performed crosslinking and immunoprecipitation experiments. In the case of the Lep signal-anchor protein,it can be seen in fig. 29 that four probe positions were tested and the TM segment indeed moved away from the translocon protein Sec61α because no crosslinking was observed from any of the four positions (lanes 9-12). To test internal TM segments probes in 211p(75) and 353p(143) were moved two amino acids away to residues 77 and 141 respectively. In the case of 211p(77) at no stage of integration was crosslinking to Sec61α or TRAM observed (data not shown). One example of 211p(77) is shown in fig. 24 (lane 8) where nascent chains of 158 residues do not crosslink to Sec61α. In the case of the construct 353p(141), the pattern of crosslinking to Sec61α was identical to that observed with the construct 353p(143) even though the probe had been moved two amino acid positions. Thus, it appears that the loss of TM1 photocrosslinking to Sec61α when TM2 enters the translocon is not due to a rotational rearrangement of TM1 within the translocon, but instead reflects the release of TM1 from the translocon.

Discussion

The experimental data in this chapter reveal an important aspect of the mechanism by which polytopic membrane proteins are threaded into the bilayer during co-translational integration into the ER membrane. The results show directly that TM segments are not released *en masse* from the translocon, as suggested by Borel and Simon (1996), nor do they support the hypothesis that the first two TM sequences to enter the translocon must also exit in a pair-wise fashion. Instead, the data support a hybrid model in which the orientation of the alternating TM segments may dictate the nature of their release from the translocon. Specifically, when a TM segment in a "stop-transfer" orientation (N-terminus lumenal) enters the translocon as the first TM segment, it remains bound to the translocon for varying lengths of time (compare Lep and VSV-G in Chapter III). In the case of VSV-G TM segments, most remain bound to the translocon until translation is terminated or until a second TM enters the translocon. But TM2 is a signal-anchor orientation (N-terminus cytoplasmic) does not leave with TM1. Instead, TM2 mostly remains in the translocon until TM3 enters the translocon, and then TM2 and TM3 leave as a pair. Although we clearly need more examples to be able to generalize the observations reported here to all membrane proteins, it appears that two TM segments with a lumenal loop can exit together, but that a pair with a cytoplasmic loop does not leave together.

Each TM sequence in a multi-spanning membrane protein was examined by placing a probe in its center and determining what was adjacent to the probe at different points during the integration of the nascent chain. It was found that the first TM sequence (N-terminus lumenal) in the membrane proteins we examined was bound by the translocon and stayed associated with translocon proteins until the subsequent TM sequence emerged from the ribosomal tunnel. The second TM sequence displaced the first TM sequence from the translocon, and TM2 stayed adjacent to translocon proteins. Importantly, this result was independent of the loop size between TM1 and TM2. TM2 (N-terminus cytoplasmic) then stayed inside the translocon until TM3 entered the translocon, at which point both TM sequences moved into the lipid bilayer. Thus, by using photocrosslinking to monitor TM segment proximity to translocon proteins, we have been able to establish experimentally that TM segments leave the translocon sequentially, either singly or in “signal-stop” pairs, during co-translational integration.

There are hundreds of substrates that use the translocon as the point of entrance into the secretory pathway and as the staging ground for membrane protein integration and assembly. The myriad of membrane proteins include proteins of varying sizes and hydrophobic stretches. Although the translocon is known to be dynamic, it seems unlikely that the translocon would be capable of maintaining a functional structure if required to hold 12 P-glycoprotein TM segments simultaneously, as is proposed by the all at once model. Since the

data in chapter III indicate that each TM segment is bound to a specific site within the translocon, the all at once model would appear to require that there be multiple nascent chain TM segment binding sites throughout the translocon that could accommodate a large number of TM domains simultaneously. The evidence presented in chapter III further suggests that TM segments bind to translocon proteins and do not significantly change their relative orientation relative to the translocon. Could a TM segment in a polytopic protein enter the translocon, bind, and then move to a different place within the translocon when other TM segments arrive? This seems unlikely, in part because it would lead to the translocon and nascent chain multi-spanning polypeptide chains getting entangled. Furthermore, when probes were moved within the TMs of 353 (data not shown), 211 (Fig. 24 lane 8 and data not shown), and 111+O2p (data not shown), no photoadducts were detected at nascent chain lengths beyond where crosslinking disappeared with the parent construct. Thus, a TM segment does not appear to move to a different binding pocket within the translocon after the subsequent TM sequence enters the translocon.

The current study places a probe at various locations within the middle of a TM segment to ascertain the proximity of that TM sequence to translocon proteins. Since we have used well-characterized TM segments in our chimeric model membrane proteins, one could argue that we are examining an artificial and non-natural situation, and that co-translational integration into the ER membrane is more complex in the cell. In support of such a viewpoint, there

are many papers in the literature that describe unexpected and unusual integration mechanisms. For example, Skach and colleagues have reported that TM1 of CFTR is inserted post-translationally after TM2 is inserted (Lu et al., 1998). Also, they have shown that proper insertion of TM8 in CFTR is dependent upon the insertion of TM7 (Carveth et al., 2002). No surprisingly, then, there are likely to be a multitude of variations on the basic, intrinsic integration mechanism. But it is the latter that we are attempting to define. Rather then tackle an unusual protein, it seemed more reasonable to us to examine well-behaved and well-studied TM segments such as those of the VSV G protein and of opsin. Once one characterizes the nature of their interactions with the translocon, then one can more rationally and fruitfully examine the more complicated substrates.

Now that the role of the translocon is known to include a direct interaction with each TM segment, studies may now focus on mechanistic issues at higher resolution. For example, the differences in the interactions between different TM sequences and the translocon in the same polypeptide may provide insight into the mechanism that any given membrane protein may use to obtain its final topology and structure. As noted above, Skach and colleagues (Carveth et al., 2002) have identified a TM sequence in the CFTR protein that depends upon the previous TM sequence for proper orientation. This observation naturally raises the question of whether successive TM sequences directly interact within the translocon. The finding that TM2 and

TM3 leave the translocon together, fig. 28, suggests that the simultaneous exit may be mediated by contact or an association between the two TM segments. One way to test this is to use fluorescence spectroscopy. One could place a donor and acceptor dye in TM3 and TM2, respectively, and monitor the extent of energy transfer during the integration process. If the two TM sequences indeed interact, then one would expect the efficiency of energy transfer to be 100% or nearly so. Alternatively, if the two TM segments are greatly separated inside the translocon, then the FRET efficiency will be much less. Another important experiment that can be done using photocrosslinking is to replace TM3 with a different TM sequence and ask whether a different pair of TM segments that are not naturally found within the same polypeptide would move out of the translocon together as opsin TM2 and TM3 did in our experiments.

Finally, two questions of timing should now be examined more closely. First, when do TM sequences obtain their final topography? It should be noted that TM2 must flip to achieve its final orientation. When does this occur? Does this timing change when followed by another TM sequence? Again, the power of fluorescence spectroscopy can be harnessed. By placing a probe at the end of the TM sequence and then measuring its accessibility to quenchers on either side of the membrane, one can determine when its final orientation is achieved. Second, when are polypeptides glycosylated and how does this affect TM sequence integration? In the dual-spanning construct 111+O2p(106), TM2 did not stay in the translocon until synthesis was complete no matter the size of the

loop between TM1 and TM2 was large. This construct also contains three glycosylation sites following TM2 that were used to confirm topography. However, it has been found by (Nilsson et al., 2003) (2003) that a nascent membrane protein that contains a cryptic glycosylation site crosslinks to the STT3 active site subunit of the oligosaccharyltransferase (OST) after passing through the translocon and no longer crosslinking to Sec61α . The above results therefore suggest that the glycosylation sites in the dual-spanning constructs in this study influence TM2 to move out of the translocon. To address this possibility, these glycosylation sites must be removed and the nascent chain length dependence of TM2 crosslinking to the translocon must be re-examined.

CHAPTER VI

SUMMARY

The results of this dissertation have revealed eight important facts about the mechanism of co-translationally integrating membrane proteins into the ER membrane. First, TM segments of membrane proteins are held in a fixed position within the translocon and cannot rotate freely. The nascent membrane protein is therefore bound to translocon proteins during integration. Second, the translocon binds TM segments oriented in either direction within the bilayer. Third, these protein-protein interactions often continue until translation is terminated. Fourth, the location of a bound TM segment within the translocon depends on the primary sequence of the hydrophobic TM sequence and appears not to be very dependent on its flanking regions. Fifth, the interaction of a TM segment with a translocon segment(s) is not required to be in a parallel fashion. Sixth, the TM segments in a multi-spanning membrane protein are released from the translocon in a sequential fashion into the lipid bilayer, exiting the translocon either singly or in pairs. Seventh, although the size of the loop between two successive TM segments can influence when a TM segment is released from the ribosome, the timing of TM segment release is primarily dictated by the entry of the next TM sequence into the translocon. Finally, the translocon does not serve simply as a gate to separate water and lipid, but is an active player that, through protein-protein interactions, regulates protein integration into the ER membrane one TM segment at a time.

The most important discovery reported here is that protein-protein interactions control both the movement and the location of the TM segment during integration and that all TM segments do not diffuse quickly from the translocon. Instead, TM segments stay in close proximity to the translocon until translation is terminated or until displaced by the next topogenic sequence in the polypeptide. The translocon proteins therefore play an active role in cotranslational integration. Although the exact nature of the nascent chain-translocon interactions have yet to be characterized, the current study reveals that future investigations must focus on protein-protein interactions to understand the molecular mechanisms that accomplish integration.

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VITA

NAME: Peter Joseph McCormick

EDUCATION: A.B. in biochemistry

December 1996

Washington University in St. Louis

St. Louis, MO

Ph.D. in biochemistry

Texas A&M University

College, Station, TX

PERMANENT ADDRESS: 1545 18th Street NW, #806

Washington DC

20036-1345

EMAIL ADDRESS: pmccorm@neo.tamu.edu